MECHANISM OF ACTION OF NITAZOXANIDE AND RELATED DRUGS AGAINST HELMINTHS.

A Thesis submitted for the Degree of Doctor of Philosophy of the University of London.

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Richard R. D. Atherton.
BSc (Hons), MSc.

Department of Infectious and Tropical Diseases,
London School of Hygiene and Tropical Medicine,
London, United Kingdom.

ABSTRACT

The 5-nitrothiazole, nitazoxanide, is a novel compound with a broad spectrum of activity; effective against anaerobic/microaerophilic intestinal protozoa and bacteria. The mechanism of nitazoxanide's anthelmintic activity is unknown. This study examined the mode of action of nitazoxanide and analogues against nematodes, trematodes and cestodes. *Caenorhabditis elegans*, a free living nematode, was used as a model. Nitazoxanide was found to exert a transient spastic paralytic effect, particularly at the posterior end of the nematode, with 50% effect at 4.62μM. This effect is similar to that of levamisole (a neuromuscular agent) specific for nicotinic receptors. An effect of nitazoxanide at nicotinic receptors was demonstrated using mutants of *C. elegans* and the specific neuroblockers, mecamylamine and pempidine. A neuromuscular effect was also seen in *Ascaris suum* at higher concentrations using electrophysiological technique. Nitazoxanide had no effect on L_3 larvae of *Nematodirus spathiger* and *Haemonchus contortus*.

Cestodes and trematodes *in vitro* showed high sensitivity to nitazoxanide with <3μM causing rapid paralysis and tegumental damage in *Hymenolepis diminuta* and *Schistosoma mansoni*. This was accompanied by inhibition of α-bungarotoxin binding to nicotinic receptors in *S. mansoni,* further suggesting an effect on neurotransmission. Uptake of glucose by the schistosome was decreased, and tegumental damage was found, suggesting that nitazoxanide can impair worm nutrition. Analysis of physicochemical properties of nitazoxanide suggested it is a protonophoric uncoupler. Consistent with this, synthesis of ATP was inhibited significantly by 33μM nitazoxanide in *C. elegans* and *S. mansoni.* In mitochondria of *H. diminuta* an increase in oxygen uptake was observed comparable to that seen with known protonophores suggesting that uncoupling of oxidative phosphorylation was occurring. In conclusion, nitazoxanide is likely to affect helminths by a direct effect on neurotransmission and protonophoric uncoupling. Nematodes are less sensitive than trematodes and cestodes *in vitro*, possibly due to difficulty of drug adsorption through the cuticle.

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LIST OF ABBREVIATIONS.

ACh = Acetylcholine.

AChase = Acetylcholinesterase.

AchR = Acetylcholine receptor.

ADH = Alcohol Dehydrogenase.

AIDS = Acquired immune deficiency syndrome.

AntiA = Antimycin A.

APF= Artificial perienteric fluid.

ASCh = Acetylthiocholine.

ADP = Adenosine diphosphate.

ATP = Adenosine triphosphate

BoP = Bodipy pirenzepine.

BSA = Bovine Serum Albumin.

BTX = Alpha-bungarotoxin.

BZNT = 2-benzamido-5-nitrothiazole.

CCCP = Carbonylcyanide m-chlorophenylhydrazone

Conc = Concentration.

CoQ = Coenzyme Q.

DEC = Diethylcarbamazine

DMEM = Dulbecco's Modified Eagle's Medium.

DNNTZ = Denitro nitazoxanide

DNTIZ = Denitro tizoxanide.

DHA = Dihydro artemisin.

DMSO = Dimethyl sulfoxide.

DNA = Deoxyribonucleic acid.

EBSS = Earle's Balanced Salt Solution.

EDTA = Ethylenediamine tetra-acetic acid

EGTA = Ethyleneglycol-bis- (ß-Aminoethyl ether) N,N,N′,N′-Tetraacetic acid.

Eo' = Midpoint Redox Potential.

FCCP = Carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone.

FCS = Foetal Calf Serum.

FDA = Food and Drug Administration.

FMN = Flavomononucleotide.

g = Acceleration due to gravity

G-6-P = Glucose-6-Phosphate.

GABA = Gamma amino butyric acid.

GTP = Guanosine triphosphate.

HPLC = High Performance Liquid Chromatography.

HYC = Hycanthone.

LEV = Levamisole.

LSHTM = London School of Hygiene and Tropical Medicine.

LUC = Lucanthone.

μM = Micromolar.

mM = Millimolar.

M = Molar.

MDBK= Mardin-Darby Bovine Kidney Epithelial.

MEB = Mebendazole.

MES= 2-[N-Morpholino]ethanesulfonic acid.

MET= Metrifonate.

MOPS = 3-[N-Morpholino]propane-sulfonic acid.

NAD = Nicotinamide Adenine Dinucleotide

NADH= Nicotinamide Adenine Dinucleotide (reduced)

NADP = Nicotinamide Adenine Dinucleotide Phosphate

NADPH = Nicotinamide- adenine dinucleotide phosphate (reduced)

NGM = Nematode Growth Medium.

NIC = Niclosamide.

NIRI = Niridazole.

NITF = Nitrofurazone.

NTZ = Nitazoxanide.

OD = Optical Density.

OLIG = Oligomycin.

OXA = Oxamniquine.

PBS = Phosphate buffered saline.

PCR = Polymerase Chain Reaction.

PIPES = Piperazine-N,N′-bis[2-ethanesulfonic acid]

PFOR = Pyruvate ferredoxin oxidoreductase.

PRAZ = Praziquantel

PROG = Proguanil.

RO11 = Methylclonazepam

ROT = Rotenone.

RQ = Rhodoquinone.

RFU = Relative Fluorescent Unit.

RhBTX = Tetramethylrhodamine α-bungarotoxin.

SEM = Scanning Electron Microscopy.

SHAM = Salicyl hydroxamic acid

THIA = Thiabendazole.

TIZ = Tizoxanide.

TIZg = Tizoxanide glucuronide.

Tris = Tris[hydroxymethyl]aminomethane

UHTS= Ultra High-Throughput Screening

VLA = Veterinary Laboratories Agency

WHO = World Health Organization.

CHAPTER 1 – INTRODUCTION.

1.1 Helminths.

1.1.1 Background.

Helminthic disease remains one of the world's most prevalent and economically important parasitoses of man and his domesticated animals (WHO, 1998). The majority of helminth infections are light and cause little morbidity, nevertheless many are so widespread that the low percentage of patients who suffer severe clinical disease still represent a problem of great medical and economic importance (Muller, 2003). Helminthiasis flourishes especially in warm environments marked by inadequate sanitation, parasitized reservoirs and vectors, and contaminated food and water sources (Hardman & Limbird, 1996). This is particularly the case in developing countries where the systems for livestock production and the environmental and socio-economic conditions are highly conducive for the development, maintenance and transmission of infection (Chowdhury & Tada, 1994). The population of these developing countries, suffer not only as a direct result of these infections but also suffer co-morbidity caused by other factors such as anaemia, malnutrition and associated reduced immunity (Grover 2001). However, affluence does not protect against helminth infections and young or debilitated individuals are particularly vulnerable, regardless of socioeconomic status (Hardman & Limbird, 1996).

Among the nematodes, it is estimated that *Ascaris lumbricoides* infects over 1.3 billion people with 250 million suffering from associated morbidity; the hookworms *Ancylostoma duodenale* and *Necator americanus* infect over 1.25 billion; *Trichuris trichiura* infects 1 billion with a high unmeasured morbidity (WHO, 1998). With regard to trematode infections, although several species may inhabit the intestinal tract of man, few are considered serious pathogens (Healy, 1970). One trematode infection of the blood however, schistosomiasis (i.e. bilharzia) is serious and widespread and one of the six diseases selected as especially significant in the World Health Organization's "Special Programme for Research and Training in Tropical Diseases," (Basch, 1991). The total number of people infected with any of the three major or several minor forms

of schistosomiasis is not known (Basch 1991). It is estimated by the WHO that 200 million people are infected, of which 120 million are symptomatic and 20 million have severe disease (WHO, 1994). Cestode infection is also important, though generally less than that of nematodes and trematodes (Vanden Bossche 1985). *Taenia saginata* and *Taenia solium* cause much economic loss amongst livestock with *T. solium* also causing the fatal disease, cysticercosis in humans (Kocięka, 1987). The estimated number of cestode infections is thought to be over 150 million globally with over 76 million infections with *Taenia saginata* alone (Peters & Pasvol, 1995), and although in the majority of cases, direct effects of the worms in the gut are not serious, side effects such as nausea and abdominal pain cause much discomfort (Kocięka, 1987). Though the vast majority of helminthic infections are carried by the populations of the developing countries of the world, *Diphyllobothrium latum*, *Taenia saginata*, *Enterobius vermicularis* and *Trichinella spiralis* still retain an impact in the developed or industrialised nations (Crompton, 1987).

Despite the huge amount of infection present in humans and animals, there is still very little understanding of helminth biology and anthelmintic pharmacology (Geary *et al*, 1999). Increased knowledge of biology and greater understanding of anthelmintic mechanisms would aid greatly in the control and prevention of infection.

Nitazoxanide (NTZ) was first described in 1975 (Rossignol and Cavier, 1975), and has since been shown to have activity against a wide range of parasitic protozoans (e.g. *Giardia intestinalis, Entamoeba histolytica, Trichomonas vaginalis*) (Adagu *et al*, 2002) and anaerobic bacteria (e.g. *Clostridium* spp) (McVay & Rolfe, 2000; Dubreuil *et al*, 1996). Its anthelmintic properties were described in 1984, and it demonstrated marked activity against *Taenia saginata* and *Hymenolepis nana* (Rossignol & Maisonneuve, 1984). The mechanism of action of NTZ against helminths as yet has not been fully investigated. An understanding of this mechanism of action would greatly aid future chemotherapy of helminthic infection. In this thesis, *in vitro* activity of NTZ and its metabolites/derivatives was investigated against a wide range of helminths including the free-living *Caenorhabditis elegans* and helminths from both the main phyla e.g. Nematoda and Platyhelminthes causing disease in humans.

1.2 Classification.

Helminth is a general term meaning worm. The helminths are invertebrates characterized by elongated, flat or round bodies and are biologically very different. There are 2 phyla of helminths, which are of medical importance; Nematoda (roundworms) and Platyhelminthes, which contains the two distinct classes, Trematoda (flukes) and Cestoda (tapeworms) (figure 1.1). The definitive classification is based on the external and internal morphology of egg, larval (juvenile), and adult stages (Castro, 2003). This classification is helpful in planning chemotherapy, since members of a particular class are frequently susceptible to the same drug type (Rakel, 1997). Knowledge of the life cycle stages is also important and is the basis for understanding the epidemiology and pathogenesis of helminth diseases, as well as for the diagnosis and treatment of infected patients (Castro, 2003).

Figure 1.1: **General taxonomic tree of helminths.**

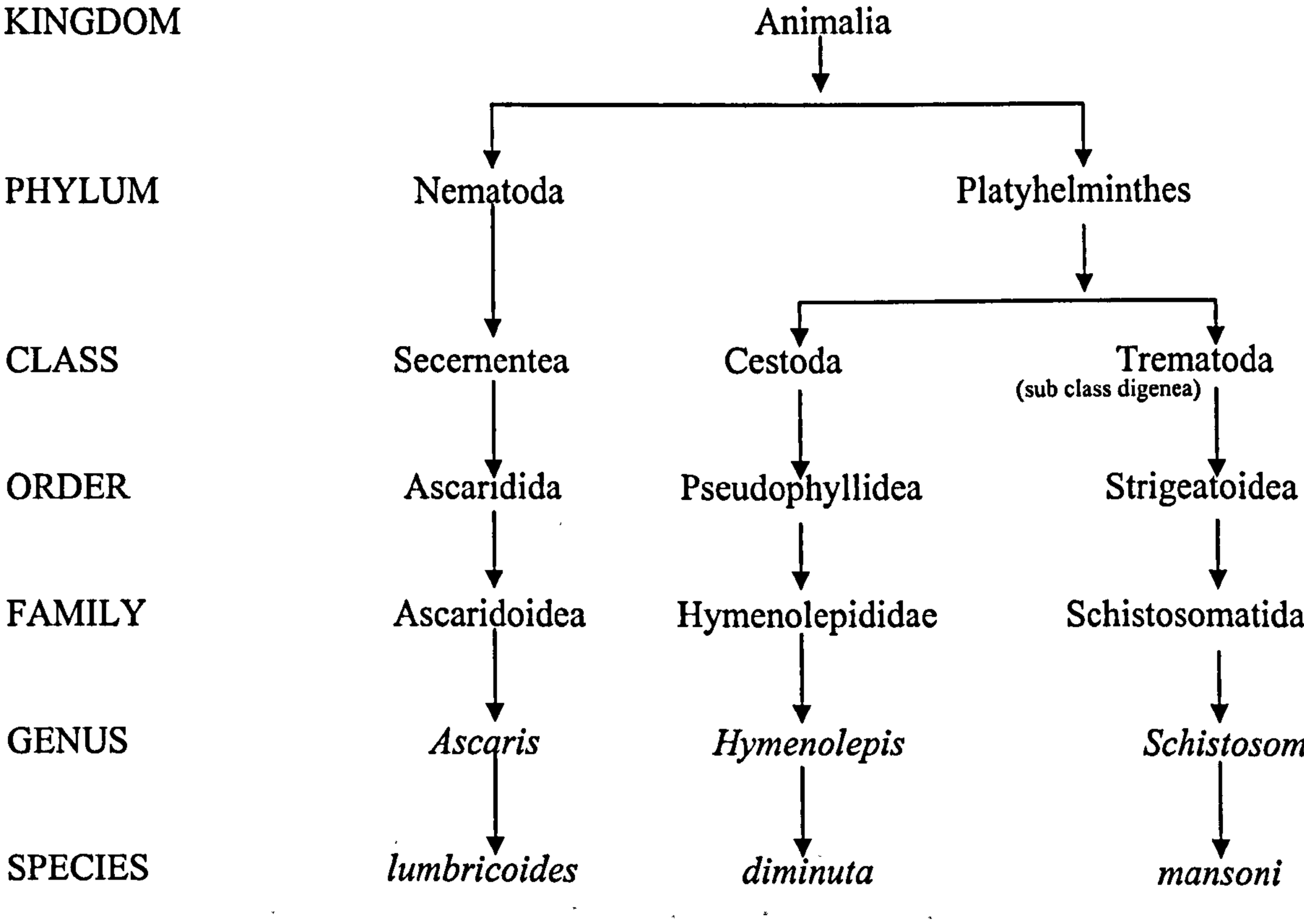


Fig 1.1: Classification of three helminths which can infect humans: One from each of the Classes Secernentea, Cestoda and Trematoda.

1.2.1 Phylum: Nematoda.

The word nematode literally means 'threadlike'. Nematodes are round worms and the oldest written record of nematodes is thought to be to the intestinal roundworm *Ascaris* in China 4,700 years ago (Maggenti, 1981). Nematodes constitute one of the largest animal phyla with some 20,000 species already classified, and can live as obligatory parasites of plants and animals, they can alternate a parasitic with a free living life style, or be strictly free-living (Broeks, 1997). All nematodes are morphologically, anatomically and developmentally very similar. The free- living nematodes occur in all aquatic and terrestrial habitats.

Nematodes are pseudocoelomate bilateralia with flexible and living (though mostly inert) cuticles and have somatic longitudinal muscles for movement (Croll & Matthews, 1977). They are usually called roundworms because they are round when viewed in cross section. They are however cylindrical in structure and taper towards their anterior and posterior ends. Nematodes have no appendages, but may have cuticular bristles or sensory setae. Their body tissues are believed to be permanently under pressure. Nematodes possess digestive, nervous, excretory, and reproductive systems, but lack a discrete circulatory or respiratory system. Nematodes have been characterized as a "tube within a tube" (Brusca *et al,* 2003); referring to the alimentary canal which extends from the mouth on the anterior end, to the anus located near the posterior end. This inner tube is located in a continuous body cavity surrounded by the longitudinal muscles of the body wall (Croll & Matthews, 1977). Their nervous system has sense organs of different modalities, central ganglia and neuromuscular junctions. Although organisation differs from the mammalian neuromuscular system, two established mammalian neurotransmitter pathways are thought to operate; acetylcholine functions as an excitatory transmitter and γ-aminobutyric acid (GABA) is inhibitory (James & Gilles, 1985). Neuromuscular function is basically similar in all nematodes but as yet, it is not completely understood (James & Gilles, 1985). This neuromuscular system is known to be a target site for a number of anthelmintics and a greater understanding would enable a more systematic search for agents that could modulate the degree of muscle function and aid expulsion of the worm from the infected man or animal.

The sexes of nematodes are typically separate, and eggs are laid which pass through four "larval" stages (L_1- L_4) before the adult stage is reached. Some nematodes however, can be hermaphroditic such as the free living *Caenorhabditis elegans*. The smallest adult species which is free-living is about 250μm long, and most species of soil, marine and freshwater forms are about 1mm long as adults (Croll & Matthews, 1977). Nematodes which parasitize are found in all parts of the body but are most commonly found in the digestive and respiratory tracts and the circulatory system. Parasites of animals/mammals tend to be 1-10mm in length e.g. *Trichostrongylus axei*, but many are bigger. *Ascaris lumbricoides* is about 20-30 cm long and *Dracunculus medinensis* (guinea worm) and *Dioctophyme renale* (giant kidney worm) are up to one metre long (Cook, 1998). The biggest known nematode species is *Placentonema gigantissima*, found in the placenta of sperm whales, which is 8 metres long.

There are a number of different groups of nematodes which infect humans. These include the geohelminths (e.g. *Strongyloides sterocoralis*, *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms), a group of intestinal nematodes, which, while not related zoologically, are all soil-transmitted and have great similarities in epidemiology and methods of control (Muller, 2003). One feature which is common to all these intestinal helminths, and distinguishes them from their hosts, is their ability to survive in an environment which is predominantly anaerobic. Here, their chief energy source carbohydrate, is metabolised by a biochemical pathway different from that utilized by the host's tissues (James & Gilles, 1985) and produces various end products depending on species e.g. 2-methylbutyrate for *A. lumbricoides* (Barrett, 1994).

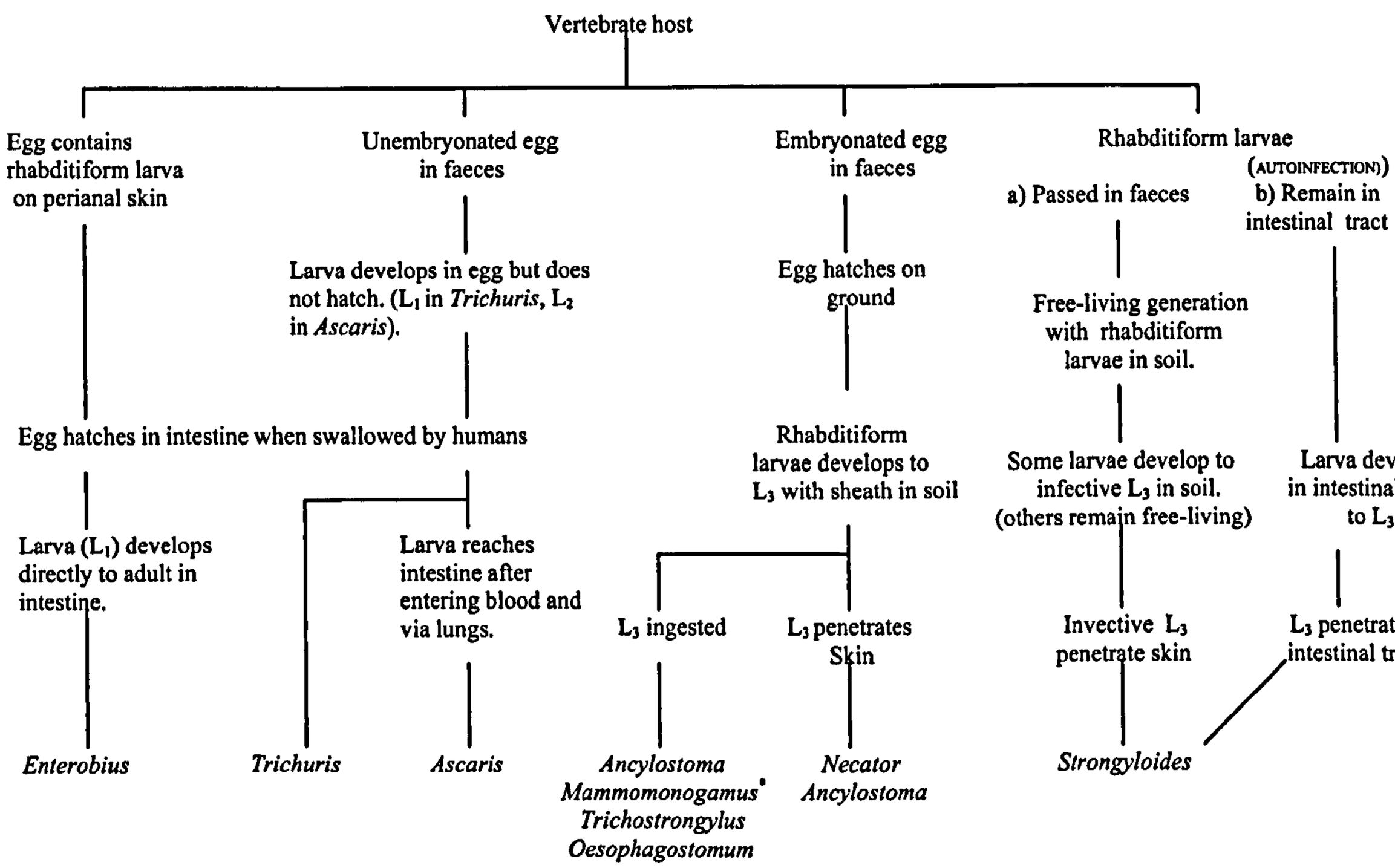
Another group consists of the tissue nematodes which includes *Trichinella* and the filarial nematodes, which invade the tissues of the host, including those of the intestine, and have a life cycle involving an intermediate host, which is either a vertebrate intermediate or an arthropod (Muller, 2003). Nematodes of the order Spirurida all have an arthropod as an intermediate host and include the filarial worms such as *Wuchereria bancrofti* which is a cause of lymphatic filariasis.

1.2.2 Transmission of nematodes.

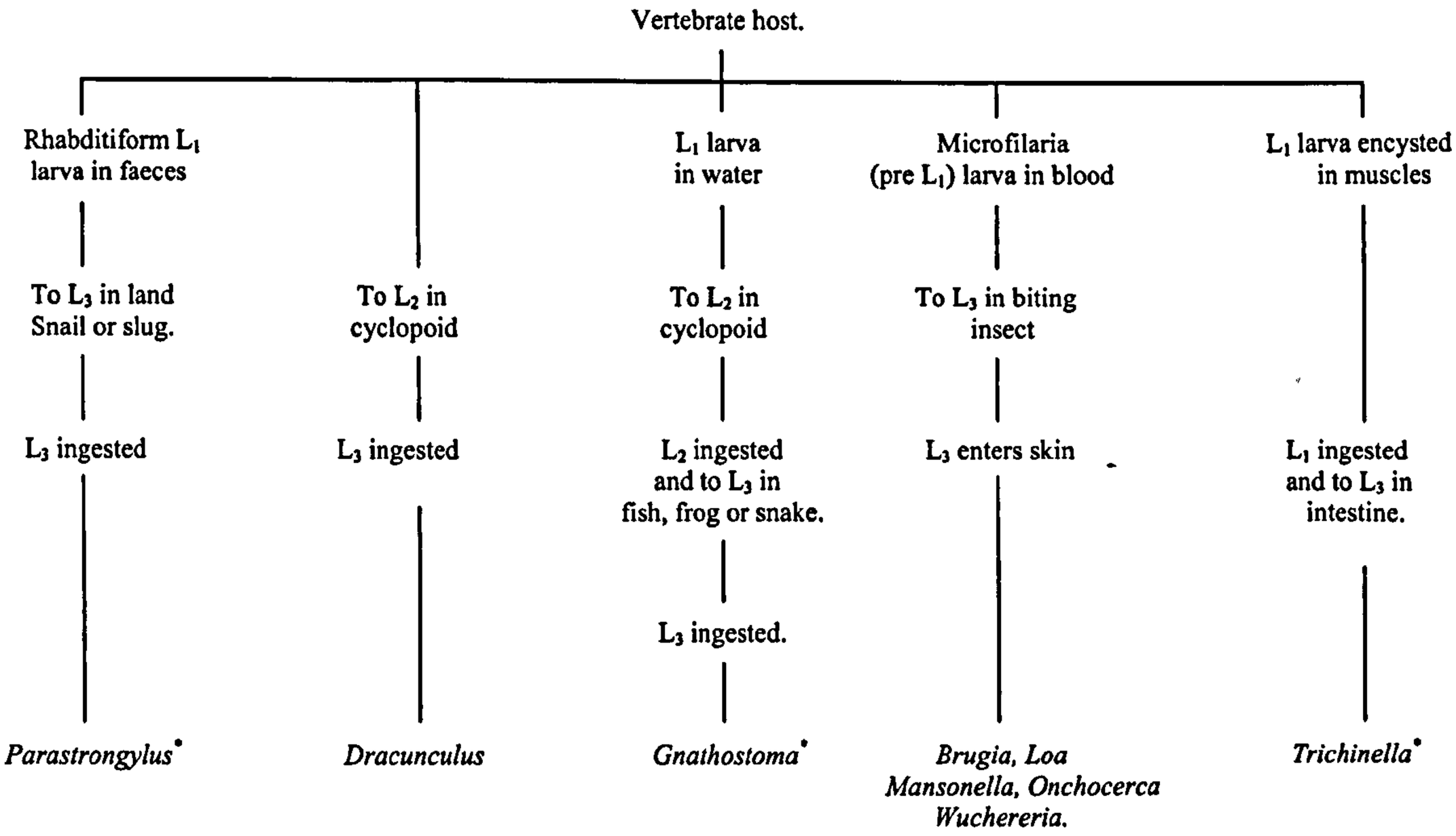
There are a number of different life cycles and hence different modes of transmission of nematodes. These life cycles are either direct (as for the geohelminths) with no intermediate host needed or they are indirect (as for the filarial worms), requiring an intermediate host (see figure 1.2 a and b).

Figure 1.2 Outline of nematode lifecycles (adapted from Muller, 2003 p110).

a) Direct cycle: no intermediate host.



b) Indirect cycle: require intermediate host.



• Humans are accidental or aberrant hosts. (For life of *Ascaris* sp see Appendix II a)

1.2.3 Phylum Platyhelminthes: Class Trematoda.

Trematodes or "flukes" are a large group of parasites, and some of those belonging to the sub-class Digenea infect man. These digenetic trematodes usually have oral and ventral suckers as holdfast organs and have an indirect and complex life cycle. Adult digenetic trematodes, with very rare exceptions are endoparasites occurring exclusively in vertebrate hosts. They should be differentiated from other subclasses of trematoda, such as the Monogenea which are usually ectoparasites of fish and occasionally endoparasites of fish and turtles, and the Aspidogastrea which are endoparasites of fish, turtles and molluscs. These show more complex holdfast organs and usually have a direct life cycle (Kumar, 1999).

The digenetic trematodes include several parasites that have an enormous impact on human populations, such as the blood flukes that cause schistosomiasis and human liver flukes (*Fasciola hepatica*). Adult flukes are typically found in the major viscera such as the bile ducts, lungs and alimentary canal; the schistosomes occur exclusively in the

blood system (Smyth, 1966). The digenetic trematodes differ from other groups of parasitic worms in that the first larval stages of all species develop in intermediate hosts from the Phylum Mollusca. This relationship is difficult to account for and one hypothesis suggests that trematodes were originally parasites of molluscs and secondarily developed an association with vertebrate hosts (Smyth, 1966). Digenetic trematodes have developed a great variety of methods for transmission from one host to another, and for survival in each (Basch, 1991).

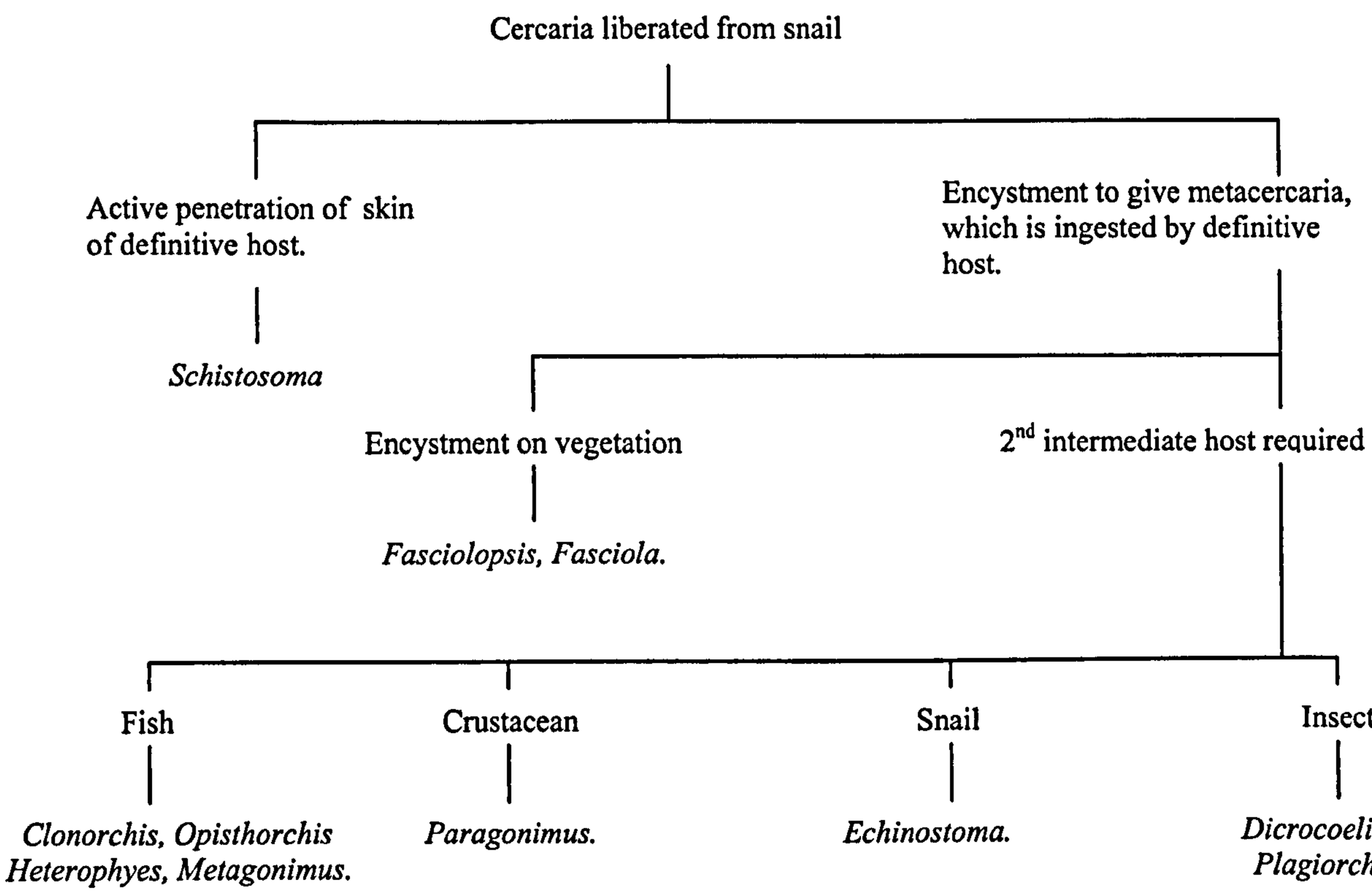
Except for the blood flukes, trematodes are hermaphroditic. The male organ consists usually of two testes with accessory glands and ducts leading to a cirrus that extends into the common genital atrium. The female gonad consists of a single ovary with a seminal receptacle and vitellaria, or yolk glands, that connect with the oviduct as it expands into an ootype where a shell is secreted around the egg. The tubular uterus extends from the ootype and opens into the genital atrium. Both self- and cross-fertilization occur (Castro, 2003).

Digenetic eggs usually exit the host with the faeces, but in some species eggs are found in the urine, sputum or elsewhere. In some species the eggs, when liberated in the environment, must be ingested by a snail, the first intermediate host. However, it is more common that the eggs hatch in the environment, releasing a swimming multicellular miracidium which locates and penetrates an appropriate mollusc in which it can develop (Basch, 1991). Within the tissues of this host the miracidium metamorphoses rapidly to a mother or primary sporocyst, Depending on species, sporocysts develop further into either a secondary, passive sporocyst or into rediae, which possess a mouth, pharynx and gut and move actively within the molluscan host. Eventually, tailed swimming larvae called cercariae are formed, which escape from the mollusc and, again depending on species, carry out a predetermined pattern of behaviour (Basch, 1991). Some, such as the liver fluke *Fasciola*, attach to aquatic vegetation (e.g. water cress), where they lose their tail and metamorphose slightly to become a metacercaria (metacercarial cyst). Some cercariae enter a specific second intermediate host e.g another snail or a fish where they develop into metacercariae. These metacercariae are either active or encysted according to species and they wait for the transitory host to be eaten by an acceptable definitive host where sexual maturity can ensue. A third pattern (followed by schistosomes), has the cercariae penetrate

directly through the skin into the body of the definitive vertebrate host (Basch, 1991) bypassing the metacercarial stage. Figure 1.3 shows the varying forms of transmission for different species.

Figure 1.3 Modes of infection of trematodes of medical importance.

Adapted from Muller, 2003 p8



(for full life cycle of *Schistosoma* sp see Appendix II b).

One of the most important features of trematodes is their tegument. The tegument is the host-parasite interface and is the outer body covering which overlays the entire parenchymal basal lamina of digenetic trematodes (Kumar, 1999). It differs from the tough and largely inert cuticle of nematodes in that it is metabolically active and has absorptive function. The tegument has been a focus of attention in recent years, because in the case of schistosomes, the tegument associated antigens have provided important clues in vaccination studies and in the case of *Fasciola hepatica*, the outer most covering of the tegument (glycocalyx), participates in evasion of the host's

inflammatory and immune assault (Kumar, 1999). The tegument also plays an important role in relation to chemotherapy. Due to its metabolic activity and ability to absorb nutrients, it may provide a good target for drug action or facilitate drug entry into the organism. For example, praziquantel damages the schistosome tegument affecting absorptive processes and possibly host evasion (De Silva *et al*, 1997).

1.2.4 Phylum Platyhelminthes: Class Cestoda.

The tapeworms are members of the class Cestoda. All adult members of this group are parasitic in the alimentary tracts, or associated ducts, of vertebrates (Vanden Bossche, 1985). Cestodes can infect man both in their adult stage (tapeworm) and larval stages (Kocięka, 1987). Man is usually infected with a single tapeworm, but multiple or mixed infections (with e.g. *T. solium*) do occur. The bodies of tapeworms lack a body cavity or alimentary system, are usually flat and elongate, and divided into three major regions; the scolex, the neck and the body consisting of a segmented chain (strobila). The scolex bears various organs of attachment such as suckers or bothridia, which assist the worms in maintaining their position in the gut. Scolices of tapeworms that infect humans are either cap-like (acetabulate) or bear several suckers (bothriate) (Bogitsh, 1998). The neck is a short area behind the scolex and is an area of very active cell division (Vanden Bossche, 1985). The strobila consists of a series of proglottids or segments. Each proglottid is a complete unit in itself and has its own set of male and female reproductive organs. In the order Cyclophyllidea, to which most of the adult tapeworms of humans belong, the proglottids vary in sexual development along the length of the strobila so that they become mature as they move further from the neck (Muller, 2003). These successively maturing proglottids eventually become gravid, when they are filled with eggs. Cestodes are hermaphrodites and they reproduce by passing out eggs contained in a gravid proglottid which detaches from the strobila. The cattle tapeworm of man *Taenia saginata* can consist of 1000-2000 proglottids with gravid proglottids containing 0.8-1 x 10^5 eggs (Muller, 2003). Eggs are ingested by an intermediate host in order to maintain transmission. Almost all cestodes have at least one intermediate host in their life cycle. The cycles vary widely among species, but generally a metacestode matures in the intermediate host, which is in turn ingested by the definitive host. Intermediate hosts include insects, crustacea, amphibians, reptiles, fish, birds and mammals (for lifecycle of *Hymenolepis diminuta*, see Appendix II c).

Different tapeworm species vary widely in size, ranging from a few centimetres (*Hymenolepis nana*) to several metres (*Taenia solium*). The outer coating of the tapeworm is the syncytial tegument, which bears numerous small projections called microtriches. The dimensions of these projections vary according to species and locations on the strobila. They resemble microvilli, but unlike typical microvilli, each microthrix includes an electron dense apical tip separated from the more basal region by a multilaminar plate. When applied to the host's intestinal epithelium these tips provide resistance to the peristaltic movement of the intestine. They also agitate intestinal fluids with worm movement thus increasing accessibility of nutrients and flushing away of waste products (Bogitsh, 1998). Covering the entire surface of the tegument is the glycocalyx, which protects the parasite from host digestive enzymes, enhances nutrient absorption and maintains the parasite surface membrane (Bogitsh, 1998). Cestodes lack an alimentary system, and due to this, the function and permeability of the tegument play an important role in determining the activity of chemotherapeutic substances used to kill these parasites.

1.2.5 Phylum Acanthocephala.

One other group of worm-like parasites are the acanthocephalans. These organisms belong to the phylum Acanthocephela and show similarities in structure to both the nematodes and the platyhelminths (Muller, 2003). Members of this phylum are all parasitic and most species are under 1cm in length. Their lifecycle usually involves an insect or crustacean as an intermediate host. The principal diagnostic character is the presence of a proboscis armed with rows of hooks which gives rise to their being called the 'thorny headed worms' (Muller, 2003). They usually parasitise such organisms as racoons, rats and fish but acanthocephalans from the genera *Moniliformis* (e.g *Moniliformis moniliformis*) and *Macracanthorhynchus* (e.g. *Macracanthorhynchus ingens*) are occasional parasites of humans.

1.3 Symptoms of helminth infection.

With over 200 species of parasitic helminth (with representatives from the Digenea, Cestoda, Nematoda and Acanthocephela) recorded as infections of man (Crompton, 1987) there is a wide range of helminth pathology. However, in the majority of cases,

helminth infection does not result in disease. Most of the helminths that are predominately human parasites are pathogenic only when worm burdens are high and, as there is no multiplication within the body, light infections only become clinically important following reinfection (Muller, 2003). As previously mentioned, helminths develop through egg, larval, and adult stages and each of these various stages (depending on species) may contribute to different disease states and pathological changes in humans (see table 1.1).

Table 1.1 Stages of helminths commonly responsible for pathologic changes in humans.

Helminths	Egg	Larva	Adult
Flukes	+++†	+*	+
Tapeworms	-	+++	+
Nematodes	-	++	+**

* Migrating and developing larval forms may cause transient pathologic responses in the host.

** Adults of *Ascaris* and filarial nematodes e.g. *Wuchereria* can cause severe pathology.

† In flukes, it is only in schistosomes where the eggs are responsible for pathology.

+++ = severe pathology

++ = moderate pathology

\+ = light pathology

Table 1.1 modified from Castro, 2003

As seen from table 1.1 each stage of the helminth life cycle can be a cause of pathology. Flukes cause pathology in all stages of their life cycle, where in the case of schistosomiasis a substantial number of eggs are retained in the tissues where they can survive for around 3 weeks (WHO, 1994). It is these eggs which are responsible for inducing most of the pathological manifestations of disease such as granulomas and pseudopapillomas (WHO, 1994). With *S. mansoni*, the eggs can cause ulceration of the

colon and can result in blood loss of up to 12.5 ml day^{-1} as seen in Egyptian patients (Muller, 2003). Schistosome infection (especially *S. japonicum*) can also cause carcinomas of the large intestine and rectum associated with the chronic lesions caused by the presence of eggs (Matsuda *et al*, 1999). In the cestode, *Taenia solium*, which is the pork tapeworm of man, inappropriate development of the larva in the definitive host, man, causes cysticercosis. Cysts may be found in the brain, which may cause epilepsy and/or other symptoms (Cook, 1998). Another cestode infection, echinococcosis caused by e.g. *Echinococcus multilocularis*, has the human as an aberrant intermediate host. Normally after infection, a cyst develops in the definitive host (e.g foxes or voles), containing protoscolices which develop rapidly into adults (Stettler *et al*, 2003). In humans however, the cysts cannot develop further, and instead cause a fibrous host reaction. The parasite destroys the liver parenchyma, bile-ducts and blood-vessels, resulting in symptoms of biliary obstruction. The cysts often metastase via the bloodstream to form new cysts, particularly in the lungs and brain. *E. multilocularis* is considered the most dangerous helminth infection in humans (Muller, 2003). With regards to pathology caused by adult helminths, hookworms (e.g. the nematode *Ancylostoma duodenale*) feed on the blood of their host and thus sap its vitality. Roundworms such as *A. lumbricoides* show a tendency for migration and accumulation and when in large numbers may cause life-threatening bowel obstruction, or a single worm may block a vital duct (Vanden Bossche, 1985). Tissue dwelling filarial nematodes cause considerable pathogenesis. Most of the severe manifestations of *Onchocerca volvulus* infection are due to the presence of microfilariae, with the adults being of secondary importance. The microfilariae can reach the cornea of the eye, and on dying cause a punctuate keratitis which can be followed by sclerosing keratitis leading to blindness (hence 'River blindness') (Muller, 2003). In contrast, in lymphatic filariasis, it is the adult worms which cause most of the pathology (Muller, 2003) e.g lymphoedema and elephantiasis.

As well as the direct pathology from the helminths, there is also a problem with coinfection. This has been demonstrated with intestinal helminths which have been reported to increase the incidence of *Plasmodium falciparum* malaria attacks and during a *P. falciparum* malaria attack, helminth-infected patients were shown to be more likely to develop anaemia and carry gametocytes (Nacher, 2002).

One important aspect of helminth infection is, as mentioned with the schistosome eggs, the host response and degree of immunopathology stimulated.

1.3.1 Immune reactions to helmintic infections.

Infection with helminth parasites generates a strong Th2 type immune response in the host (Lynch, 1987) and a cytokine profile dominated by IL-4, IL-5, IL-6, IL-9, IL-10 and IL13 (Holland & Kennedy, 2002). The characteristic features of this response include intestinal mastocytosis, eosinophilia and goblet cell hyperplasia (Chowdhury & Tada, 2001). The nature and degree of the immune response elicited by infection are not only dependent on the level and frequency of infection and the characteristics of the parasite concerned, but they are also influenced by the location of the parasite (Muller, 2003). Helminths occupy a wide variety of habitats in the host's body, and the capacity of these to respond to infection and the nature of the response expressed can differ quite markedly.

1.4 Diagnosis of helminth infection.

For the laboratory diagnosis of helminth infection, the detection of eggs in the faeces or urine is still the most widely used method (Muller, 2003). Where eggs are less numerous, concentration methods such as Kato-Katz or sedimentation techniques can be used. Other methods include numerous immunological tests with ELISA and immunoblotting techniques. With filarial infection, routine diagnosis involves finding microfilariae, usually in stained thick blood films (Muller, 2003). Clinical diagnosis is mostly parasite-specific with certain symptoms characteristic to certain parasites.

PCR is also used for diagnosis, and is very highly specific for identification of infection. However costs of equipment and the need of staff training and infrastructure make this a less utilisable form of diagnosis in developing countries.

1.5 Chemotherapy of helminths.

1.5.1 Economics.

Eradication of helminthiasis is highly unlikely, due to its close association with human poverty. Although helminth infection is both common and cosmopolitan, its subtle clinical course generally encourages neglect until overt disease is obvious (Hardman & Limbird, 1996). Until effective vaccines become available, chemotherapy provides the single most efficient, practical, and relatively inexpensive strategy to control helminth infections (Hardman & Limbird, 1996). The selective toxicity of antiparasitic agents is based upon the parasite location, differences in host and parasite metabolic pathways, or upon the concentration of the drug reaching the parasites (Docampo, 2002). Parasite life cycle stages may have different drug susceptibilities. Antiparasitic drugs should ideally be safe, orally effective, curative in a single dose and inexpensive (Docampo, 2002).

1.5.2 Selective toxicity.

In contrast to viruses, bacteria, fungi and tumours, helminths parasitize (often simultaneously) virtually all host tissues, including blood (*Dirofilaria immitis*, *Schistosoma spp*), other tissues (*Dictyocaulus viviparous* (lung worm of cattle), *Fasciola hepatica*) and the gastrointestinal tract from stomach to anus (*Ascaris lumbricoides*, *Enterobius vermicularis*). Broad-spectrum anthelmintics must reach sufficient concentration in each of these compartments, preferably using a single delivery strategy (Geary, 1999). In the last 50 years specific, safe and effective anthelmintic drug therapies for various parasitic infestations have been developed. Earlier anthelmintic drugs suffered from serious drawbacks such as hepatotoxicity and required specific preparation of the patient before treatment. However, successive discoveries were born out of rational approach that contributed to the effective, more specific and more easily tolerated drugs (Grover 2001).

1.5.3 Current anthelmintics.

A wide range of clinically effective antihelmintics exists, creating a relatively satisfactory chemotherapeutic situation (Harder, 2002). Virtually all the important helminth infections of man can be treated with one of five main anthelmintics which are in use at the moment: albendazole, mebendazole, diethylcarbamazine (DEC), ivermectin and praziquantel. These drugs not only treat individual infections, but also aid in controlling the transmission of some of the more common infections (De Silva *et al*, 1997).

However, some helminthic diseases are still lacking effective chemotherapy such as fascioliasis and neurocysticercosis. There is also a severe lack of available anthelmintics which are able to target species from both of the two main phyla. The introduction of a new broad-spectrum anthelmintic which has activity against nematodes, trematodes and cestodes would greatly aid in prevention and control of infection.

1.5.3.1 Intestinal nematodes.

The treatment of intestinal nematode helminth infections, at both the individual and community levels is currently best served by the benzimidazoles (e.g. albendazole, mebendazole and thiabendazole) with alternative treatments using levamisole hydrochloride or pyrantel pamoate (WHO, 1987; Katz, 1977). The cure rate with these drugs is also high e.g. thiabendazole produces a cure-rate of 98% in cutaneous larva migrans while mebendazole gives cure rate of 76-95% in ascariasis, trichiuriasis and hookworm infestations (Grover, 2001). Piperazine salts though cheap, are only useful for ascariasis and enterobiasis. The efficacy of single dose therapy in the treatment of intestinal nematode infestations has made feasible mass treatment programmes targeted at school children, the age group with the highest prevalence. Such mass treatment has been advocated as a component of control measures to reduce the number of worms in individual children below pathogenic levels and has been shown to improve children's growth and physical fitness as well as academic performance (De Silva *et al*, 1997; Stephenson *et al*, 2000).

1.5.3.2 Filarial nematodes.

Currently, diethylcarbamazine (DEC) and ivermectin are used in the treatment of filariasis. Albendazole is also used in combination with DEC (Kshisager *et al*, 2004; Rajendran *et al*, 2004). Mass treatment with an annual dose of ivermectin has now been established as the treatment of choice for onchocerciasis. This has been shown to be effective in controlling ocular lesions and in reducing transmission, and is safe enough to use in large scale control programmes. Long-term, low dose mass treatment through the substitution of normal salt with DEC-fortified table/cooking salt has also been useful in the control of lymphatic filariasis. This has been shown to reduce microfilariae by 99% for at least one year, when used for 9-12 months (De Silva et al, 1997).

1.5.3.3 Cestodes.

Praziquantel and niclosamide are accepted as very safe anticestodal drugs effective in clinical use (WHO 1987), and between them have good activity against most human cestode infections (Cook, 1998). Mebendazole is also used as an anticestodal drug, and inhibits the growth of *E. multilocularis* cysts and prevents the occurrence of metastases (Muller, 2003).

1.5.3.4 Trematodes.

Praziquantel is the best schistosomicidal agent available today. It is the one drug effective against all species infecting man and has a 96% cure rate. There is also oxamniquine for *Schistosoma mansoni* and metrifonate, used predominately in treatment of *Schistosoma haematobium* even though it has slight action against *Schistosoma japonicum* and *Schistosoma mansoni* (Jordan, 1993). Praziquantel also has activity against *Chlonorchis sinensis* and *Paragonimus westermani* (Terada *et al*, 1982). Praziquantel and albendazole are said to be of value in the treatment of neurocysticercosis, for which there was no effective chemotherapeutic agent until 1979 (De Silva *et al*, 1997) and are also active against *Echinococcus granulosus* (Urrea- Paris et al, 2000). Until recently the only available compound for treating human fascioliasis caused by the trematode *Fasciola hepatica*, has been triclabendazole (Coles, 1986) which has only recently been introduced in Egypt (el-Karaksy *et al*, 1999). However this drug was shown to require repeated courses of treatment.

Table 1. 2 Mode of action of the major groups of anthelmintics

Anthelmintic group	Examples	Mode of Action
Macrocyclic lactones (Macrolides)	Ivermectin Eprinomectin Doramectin Moxidectin Milbemycin oxime Selamectin	Bind to glutamate chloride channels causing paralysis.
Benzimidazoles	Thiabendazole Mebendazole Fenbendazole Oxfenbendazole Oxibendazole Albendazole	Inhibition of polymerization of microtubules
Pro- benzimidazoles	Febantel	Same as benzimidazoles
Imidazothiazoles	Levamisole Tetramisole	Cholinergic agonists.
Tetrahydropyrimidines	Morantel Pyrantel	Cholinergic agonists
Organophosphates	Dichlorvos Haloxon Trichlorofon	Inhibitors of cholinesterases
Piperazines	Piperazine salts	Anticholinergic action-block neuromuscular transmission.
Uncouplers (protonophores)	Niclosamide	Uncouples mitochondrial respiration from energy production.

Table modified from "Parasites and Parasitic Diseases of Domestic Animals"
Dr. Colin Johnstone (principal author)

1.6 Anthelmintic drug mechanisms.

As presented in table 1.2, anthelmintics vary in their mechanism of action. Some show good activity against certain species of worms, yet have very poor activity against others. The general drugs for anthelmintic use are either cholinergic agonists, protonophores or anti-tubulin:

1.6.1 Anti-nematodal drugs.

1.6.1.1 The cholinergic agonists.

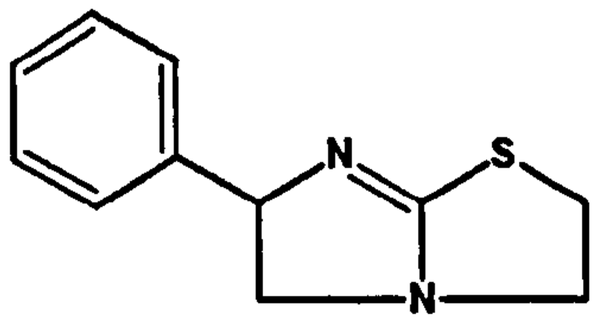
The cholinergic agonists e.g. levamisole, pyrantel (Martin, 1993; Martin *et al*, 1996), and morantel (Evans & Martin, 1996) are anthelmintics which target neurotransmitter-gated-ion-channels in the worms. These neurotransmitter-gated ion-channels provide the molecular basis for rapid signal transmission at chemical synapses. They are post-synaptic oligomeric transmembrane complexes that transiently form an ionic channel upon binding of a specific neurotransmitter (Stroud *et al*, 1990).

A number of these neurotransmitter-gated receptors present in helminths include the nicotinic acetylcholine receptor (AchR), the muscarinic AchR and the gamma amino butyric acid (GABA) receptor. The motor neurons of invertebrates, are unmyelinated and are thus more susceptible to disturbances of nerve membranes caused by membrane active agents than are the myelinated somatic motor fibers of vertebrates (Docampo, 2002). Acetylcholine receptors have different properties in different anatomical locations (Goldstein *et al*, 1974). In the neuromuscular junction or ganglia of the autonomic nervous system they are known as 'nicotinic receptors' because they are stimulated by nicotine. In smooth muscle AchR's are known as 'muscarinic' because they are stimulated by muscarine (Bacq, 1975). The nicotinic AchRs are ligand- gated ion channels that produce an increase in Na^+ and K^+ permeability, depolarisation and excitation upon activation (Haugland, 1998). Thus binding of a cholinergic agonist such as levamisole at the nicotinic receptor site would stimulate muscular contraction, causing spastic paralysis and resulting in expulsion of the worm from the host. Nicotinic receptors are selectively blocked by agents such as tubocurarine and muscarinic receptors are selectively blocked by agents such as atropine (James & Gilles, 1985). The GABA receptor is an inhibitory chloride channel receptor, which when stimulated by GABA (which acts as a transmitter substance of inhibitory neurons), prevents contraction by making the membrane of the muscle fibres permeable to chloride ions. This permeability increase reduces the effectiveness of the action of motor nerves and effectively inhibits contraction leading to flaccid paralysis of the worm (Bacq, 1975).

1.6.1.2 Levamisole.

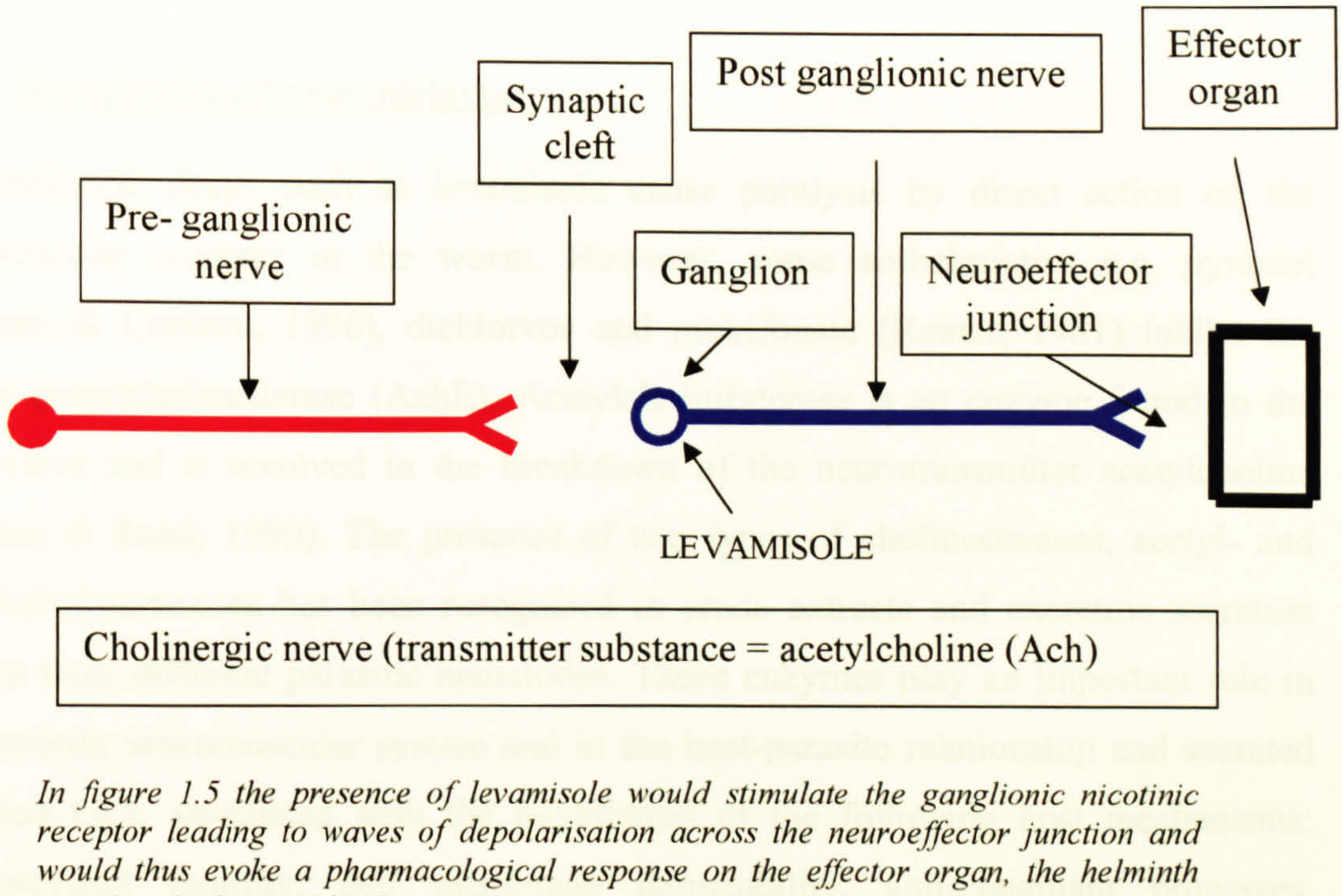
Levamisole is a potent nematocide (Richmond & Jorgensen, 1999), discovered along with tetramisole during a drug development programme in the early 1960's. Tetramisole is the parent compound and is a racemic mixture with the S(-) isomer, levamisole, containing all the biological activity. The spectrum of this drug against intestinal nematodes is narrower than that of the benzimidazoles (James & Gilles, 1985), though levamisole has very good activity against *Ascaris*. The neuromuscular mode of action of levamisole is similar to that of morantel and pyrantel, however, levamisole also has slight fumarate reductase inhibitory action, though this is considered too low to be operative *in vivo* with an anthelmintic dose regimen (James & Gilles, 1985).

Figure 1.4 Structure of Levamisole.



Neuromuscular drugs with the same or similar mechanisms when used on nematodes can produce a number of effects. On exposure to levamisole *in vitro*, immature and adult worms of *Ascaris lumbricoides* show spastic contraction followed by tonic paralysis (Martin *et al*, 1997). The drug is immediately and almost completely absorbed via a trans-cuticular mechanism (Vanden Bossche, 1985). Studies on the mode of action of levamisole indicate it is a selective agonist at acetylcholine receptors present on the muscle cells of parasitic nematodes (Robertson & Martin, 1993). Levamisole, produces a characteristic depolarising type of neuromuscular blockade. This causes an excitatory, spastic effect on the worm, which closely resembles that of ganglionic stimulation in autonomically innervated mammalian systems (figure 1.5).

Figure 1.5 Schematic representation of ganglionic stimulation by neuromuscular agonist (levamisole).



In figure 1.5 the presence of levamisole would stimulate the ganglionic nicotinic receptor leading to waves of depolarisation across the neuroeffector junction and would thus evoke a pharmacological response on the effector organ, the helminth muscle, which would lead to a transient spastic paralysis of the worm.

The excitatory effect of levamisole is similar to that of nicotine and is also abolished by the specific ganglionic–blocking agents pempidine and mecamylamine (Tomoe *et al*, 1996). It is this observed effect which led to a ganglion-stimulating type of effect being proposed for the mechanism of levamisole and other anthelmintics including pyrantel and bephenium. Pyrantel is similar to levamisole in that it causes a neuromuscular blockade and induces a marked, persistent activation of the nicotinic receptor, which results in spastic paralysis of the worm (Hardman & Limbird, 1996). Pyrantel however, also inhibits cholinesterase and it can cause a slowly developing contracture of preparations of *Ascaris* at 1% of the concentration of acetylcholine required to produce the same effect (Hardman & Limbird, 1996).

Other receptors in helminths, which include the muscarinic and GABA receptors, are targets for a wide range of drugs. GABA receptors are targets for piperazine and ivermectin, and like GABA, piperazine activates chloride channels in the membrane.

The effect of piperazine on the nematode muscle thus resembles that of inhibitory nerves, which causes worm muscles to become flaccid and thus results in worm expulsion from the host (Bacq, 1975).

1.6.1.3 Acetylcholinesterase inhibition.

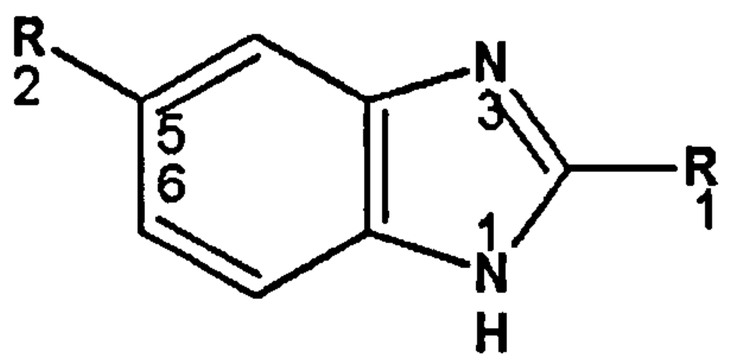
As mentioned, drugs such as levamisole cause paralysis by direct action on the neuromuscular receptor in the worm. However, some anthelmintics e.g. pyrantel (Hardman & Limbird, 1996), dichlorvos and metrifonate (Reiner, 1981) inhibit the enzyme acetylcholinesterase (AchE). Acetylcholinesterase is an enzyme found in the nerve tissue and is involved in the breakdown of the neurotransmitter acetylcholine (Bowman & Rand, 1990). The presence of two types of cholinesterases, acetyl- and butyryl-cholinesterases has been recognised in crude extracts and excretion-secretion products from different parasitic nematodes. These enzymes play an important role in the nematode neuromuscular system and in the host-parasite relationship and secreted AchE has been associated with the modulation of the following host mechanisms: gastrointestinal motility, cell membrane permeability, anti-coagulant processes, anthelmintic resistance, immune and inflammatory responses (Ros-Moreno *et al*, 2002). Drugs such as metrifonate and pyrantel, exert their effect by inhibiting the effect of helminth acetylcholinesterase and hence prevent the breakdown of acetylcholine, thus allowing a build up of acetylcholine which causes a depolarisation of the postsynaptic membrane (Bowman & Rand, 1990). Eventually the build up of acetylcholine exceeds a threshold level leading to propagated depolarisation (Goldstein *et al*, 1974), which causes paralysis and hence expulsion of the worm. The inhibition of helminth cholinesterases is likely to have an affect on the host-parasite relationship, though most of the reasons why the worm secretes cholinesterases have not yet been systemically investigated (Hussein et al, 1999).

1.6.1.4 Benzimidazoles

In 1964 it was discovered that thiabendazole (2-(4'-thiazolyl) benzimidazole) possessed a broad spectrum of activity against gastro-intestinal worms, and this opened up a new era in the treatment of parasitic diseases (De Silva *et al*, 1997). The benzamidazoles which include cambendazole, oxibendazole, albendazole and mebendazole are all

potent, orally active, broad-spectrum anthelmintics (McCracken *et al*, 1982). They have in common a bicyclic ring system in which benzene has been fused to the 4- and 5-position of the heterocycle (imidazole) (De Silva *et al*, 1997).

Fig 1.6 Generalized structure of the Benzimidazoles.



Several thousand benzimidazoles have been screened for anthelmintic activity, but only mebendazole and albendazole are currently in wide clinical use, although thiabendazole, flubendazole and triclabendazole are in use but on a much smaller scale (De Silva *et al*, 1997).

1.6.1.5 Mode of action of benzimidazoles.

The mode of action of the benzimidazole drugs has been extensively reviewed (Lacey, 1990). Mebendazole selectively inhibits glucose uptake in nematodes and cestodes which leads to increased utilization of parasite glycogen, therefore depriving the parasite of its main energy source (James & Gilles, 1985). Mebendazole and other benzimidazole drugs have the ability to bind to tubulin (Bughio *et al*, 1994; Friedman & Platzer, 1980) and act on nematodes by preventing ß- tubulin polymerising into microtubules (Lacey *et al*, 1988; Barrowman *et al*, 1984). This effect induces the disappearance of cytoplasmic microtubules (important for intracellular transport) of the tegumental or intestinal cells of cestodes or nematodes causing degenerative changes in these cells (Vanden Bossche *et al*, 1985). This may lead to impaired coating of the membranes, followed by a decreased digestion and absorption of nutrients (Rahman and Bryant, 1977), eventually leading to death of the parasite. Mebendazole has no effect on blood glucose concentrations in humans, and examination of the intestine and other

organs of treated animals has shown an intact microtubular system and normal subcellular organelles. The presence of food in the digestive tract of the definitive host does not affect the action of the drug during treatment of intestinal helminthic infections (Rahman and Bryant, 1977).

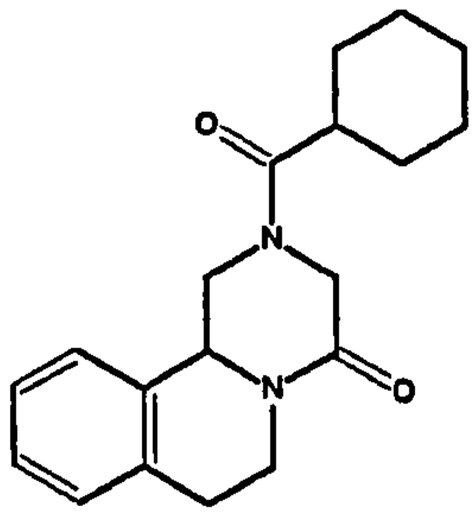
1.6.2 Anti-trematodal drugs.

There are few drugs currently used to treat trematode infection. These drugs include triclabendazole, clorsulon and praziquantel, with the last being the drug of choice.

1.6.2.1 Praziquantel.

Praziquantel is a heterocyclic pyrazino-isoquinoline and was developed after this class of compound was discovered to have anthelmintic activity in 1972 (Hardman & Limbird, 1996). Praziquantel is the best schistosomicidal agent available today for the clinical management of schistosomiasis. It is highly effective against all species of schistosomes pathogenic to humans (Webbe & James, 1977). It has also been reported to be useful in combination with albendazole or alone in the treatment of other trematode infections, chlonorchiasis, paragonimiasis and fascioliasis (De Silva *et al*, 1997), as well as the cestode infections, taeniasis and hymenolepiasis. Nematodes are unaffected (Hardman & Limbird, 1996). More than 80% of the drug is absorbed after oral administration and peak plasma levels are reached in 3-4 hours (De Silva *et al*, 1997). Praziquantel crosses the blood-brain barrier, reaching CSF concentrations approximately 25% that of plasma levels.

Figure 1.7: Structure of praziquantel.



1.6.2.2 Mechanism of praziquantel.

The mechanism of action of praziquantel remains unclear and is still under debate (Hardman & Limbird, 1996). The uptake of praziquantel is rapid and reversible (Hardman & Limbird, 1996) and effects of praziquantel on adult worms include vesiculation, vacuolisation and disintegration of the tegumental surface (Bricker *et al*, 1983; Matsumoto, 2002). Firstly at low concentrations, praziquantel causes a muscle contraction in about ten minutes, followed by subsequent flaccid paralysis (Blair *et al*, 1992). This would cause affected worms to detach from host tissues *in vivo* (Hardman & Limbird, 1996). The authors attributed this muscle contraction to a drug induced interference with the inorganic Mg^{2+} ion mechanism of the parasite. At higher concentrations, praziquantel causes tegumental damage, which activates host defence mechanisms and results in destruction of the worms (Hardman & Limbird, 1996). In schistosomes praziquantel is shown to stimulate Ca^{2+} influx (Kumar, 1999), which may cause muscle cells to contract (Coles, 1979). The drug then causes vacuolation of the syncytial tegument (blebbing), which is followed by disintegration of the surface of the tegument leading to parasite death (Kumar, 1999).

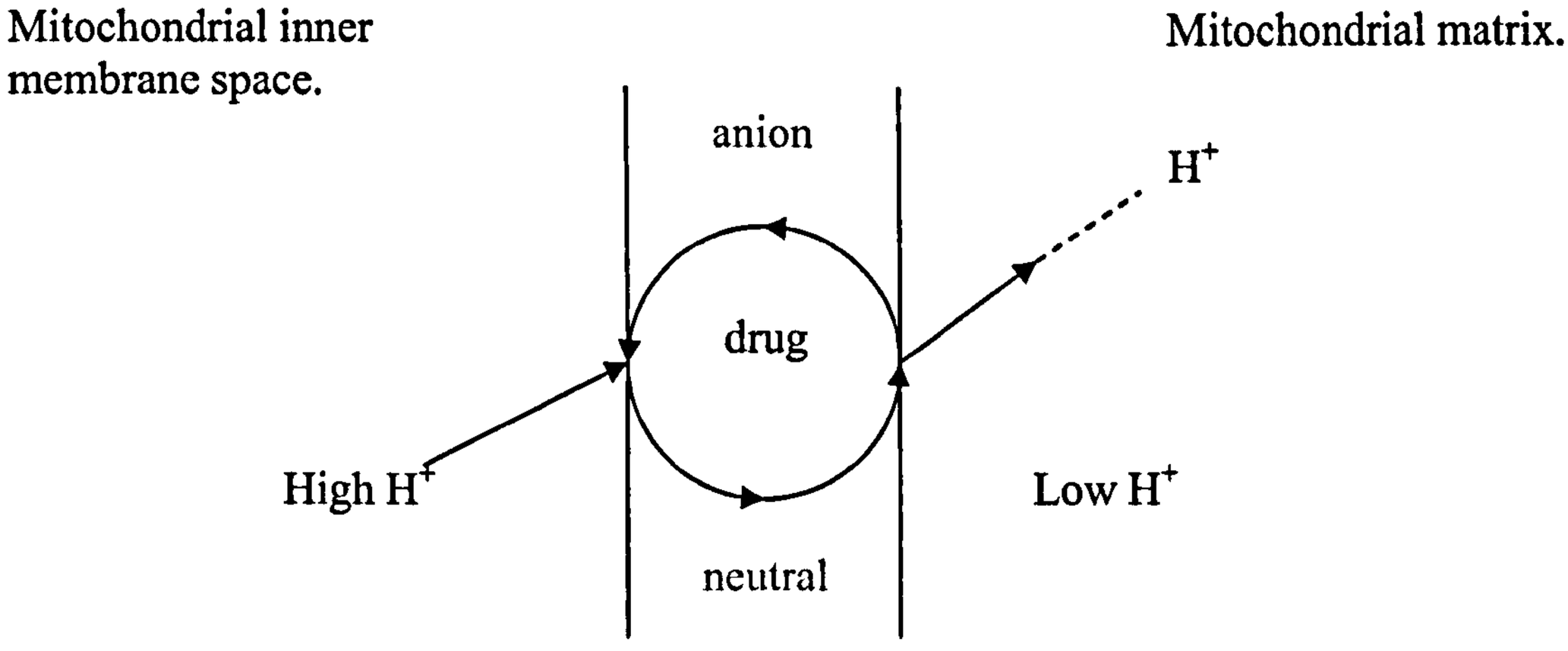
1.6.3 Anti-cestodal drugs.

Praziquantel is also the drug of choice for treatment of cestodes infection e.g. *Taenia* infections, *Diphlylobothrium latum* and *Hymenolepis* infections (Muller, 2003). However, niclosamide which has protonophoric (uncoupling) activity (Vanden Bossche *et al*, 1985) is also used.

1.6.3.1 Protonophoric activity

An uncoupler (protonophore) is a membrane- soluble small molecule with at least one weakly acidic group (-OH or >NH) which can readily release or bind a proton (Heytler, 1979). As early as 1961, it was recognised that lipid-soluble weak acids can traverse a membrane both in protonated form (R-OH, R=NH) and as the anion (RO^-, $R=N^-$), thereby effectively translocating protons across the membrane (figure 1.8) (Harold, 1986).

Figure 1.8: Proton conducting uncoupler system.



When an uncoupler dissolves in the bacterial or mitochondrial inner membrane, it can ferry protons back along the proton gradient into the matrix, bypassing the ATP-synthetic machinery. This is the mechanism by which 2,4-dinitrophenol, niclosamide and other uncouplers dissociate the linkage between processes that generate the proton potential and those that consume it. Uncouplers cause the membrane to become specifically permeable to protons and to no longer be able to sustain a proton potential (Harold, 1986). The conversion of ADP to ATP is prevented and respiratory electron transport is effectively freed from dependence on ADP supply. This leads in many cases to an increase in oxygen uptake due to more rapid respiration uncontrolled by ADP availability. The effect can be monitored by examining phosphate exchanges in mitochondria and also oxygen exchanges between inorganic phosphate, water and ATP. One uncoupler which is in current use is niclosamide (2′,5-dichloro-4′-nitrosalicylanilide). This is an anti-tapeworm drug, which is hardly absorbed through the intestinal wall of the host (Vanden Bossche, 1985).

1.7 Enzyme action.

Enzyme inhibition is another mode of action displayed by some anthelmintic drugs e.g. inhibition of the *Haemonchus contortus* fumarate reductase system by thiabendazole (Prichard, 1970). The understanding of parasite enzymes holds much potential in the finding of new drugs, as a number of target enzymes exist that are found only in the parasites. One enzyme is pyruvate ferrodoxin oxidoreductase which is found in the

protozoa *Trichomonas*, *Entamoeba* and *Giarda* spp (Docampo, 2002). This enzyme, can achieve a very negative reduction potential, and can reduce the nitro group of metronidazole to form cytotoxic reduced products that bind to DNA and proteins (Docampo, 2002). Other enzymes that are important in the mode of action of anti-parasitic drugs include acetylcholinesterase (1.6.3), nitroreductase and fumarate reductase.

1.7.1 Fumarate reductase.

The fumarate reductase system functions in the respiratory chain in intestinal helminths (Prichard, 1973). In intestinal helminths living under relatively anaerobic conditions, there is a microaerophilic reducing system in the electron transport chain, which uses fumarate (redox potential approximately +0.025mV (millivolts)) as a hydrogen acceptor instead of oxygen (Kuramochi et al, 1995). Coenzyme Q (CoQ) is a lipid soluble factor that transports electrons and protons across the inner mitochondrial membrane to maintain the proton gradient that drives ATP synthesis (Tatar *et al*, 2002). CoQ drives electron transport at complexes I and III (see figure 1.9). Worms endogenously synthesise the most prevalent Q_9 isoform to obtain CoQ from a demethoxy-Q_9 (DMQ_9) intermediate (some worms i.e. *C. elegans* can also obtain CoQ from feeding on bacteria that synthesise Q_8), they are also able to produce RQ_9 (an alternative quinone) which is involved in anaerobic respiration rather than aerobic respiration (Tatar *et al*, 2002). This allows the worms to follow an alternative system where fumarate is reduced to succinate and allows phosphorylation of ADP (Barrett, 1994).

The fumarate reductase system appears to be a particularly vulnerable point, in the metabolism of helminths, for chemotherapeutic interference (Prichard, 1973; Barrowman *et al*, 1984). This is due to the specificity of the system to the helminth in the host-parasite relationship. Many drugs have been tested for fumarate reductase inhibitory activity, cambendazole and l-tetramisole inhibit the fumarate reductase system in *Haemonchus contortus* (Malkin *et al*, 1972, Vanden Bossche *et al*, 1969). The veterinary anthelminthic, thiobendazole, inhibits fumarate reductase in *Haemonchus contortus*, *Fasciola hepatica* and *Hymenolepis diminuta* (Coles, 1977). However, the role of fumarate reductase inhibition nowadays is not widely considered to be

important, though the further understanding of respiration in nematodes is still a major factor regarding chemotherapeutic targets (Barrett, 1994).

Figure 1.9 The electron transport chain.

Diagram from Tatar et al, 2002

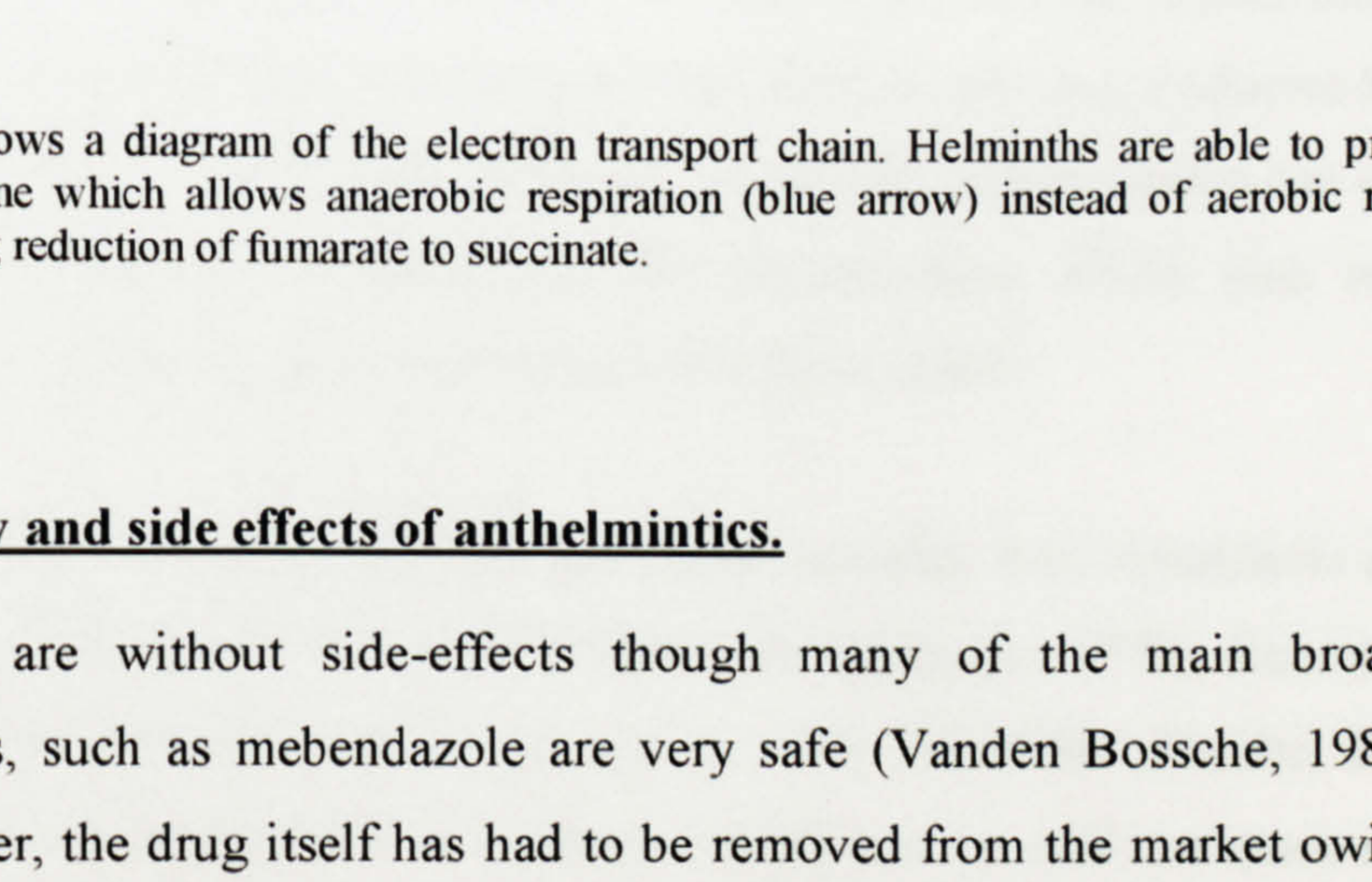


Figure 1.9: shows a diagram of the electron transport chain. Helminths are able to produce an RQ_9 alternate quinone which allows anaerobic respiration (blue arrow) instead of aerobic respiration (red arrow) allowing reduction of fumarate to succinate.

1.8 Toxicity and side effects of anthelmintics.

Few drugs are without side-effects though many of the main broad spectrum anthelmintics, such as mebendazole are very safe (Vanden Bossche, 1985). In some cases however, the drug itself has had to be removed from the market owing to severe complications. This was the case with the schistosomicidal drug, niridazole (Bassily *et al*, 1979) which was found to be mutagenic, affecting reproductive systems and was carcinogenic in animals (Frohberg, 1989). From the toxicological point of view, praziquantel is the most promising drug, because it lacks systemic toxicity after repeated administration of daily doses of up to 100 or 180 mg/kg to rats and dogs, respectively. It does not affect reproduction, and is devoid of any mutagenic or carcinogenic potential (Frohberg, 1989).

1.9 Drug resistance.

The efficacy of these anthelmintic drugs and their ability to cure disease and decrease morbidity have been well established. However in animals, where anthelmintics are used intensively, resistance is an increasing problem and has already been shown in some of the broad-spectrum anthelmintics.

1.9.1 Drug resistance in animals.

In the veterinary field, the number of reports on resistance against anthelmintics in nematodes has increased dramatically and resistance has been found in *Fasciola*, cyathostomes, ascarids, hookworms and strongyles (Conder and Campbell, 1995). In sheep, resistance was found in the trichostrongylids; *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Oesophagostomum circumcincta* and demonstrated for all the benzimidazoles commercially available (Vanden Bossche, 1985). Recently resistance to macrocyclic lactone (ML) anthelmintics e.g. ivermectin for the control of trichostrongylid larvae has become a serious problem in sheep and goats (Prichard, 2002). These parasites are prolific breeders, resulting in a huge population size within a farm. This huge population size and high reproduction rate are conducive to a high level of genetic diversity. As a result, repeated use of ML anthelmintics can select for rare individual nematodes able to survive the normal dose. These then reproduce and generate a new resistant strain of parasites (Prichard, 2002)

Resistance in animal helminths has also been recorded with levamisole and morantel, drugs with a similar mode of action to each other (Sangster 1979). Recently ivermectin resistance has been seen to be on the rise in cattle helminths (Harder, 2002) and this demonstrates the urgent need in veterinary medicine for new anthelmintics with new modes of action (Harder, 2002). This emergence of anthelmintic resistance and associated economic problems in several animal industries has raised much concern, and highlights possible threats to the control of human parasites (Sangster, 1999).

1.9.2 Drug resistance in humans.

To date there is no confirmed report of anthelmintic drug resistance in a soil-transmitted nematode infection in humans (De Silva *et al*, 1997). However, the detection of resistance is difficult, unless acute disease is a feature of the infestation, so most helminth resistance is likely to go unrecognised (Chowdhury & Tada, 1994). The widespread use of mebendazole and albendazole for treating intestinal nematode infections in human populations is raising concerns that careful monitoring procedures should be in place to identify any emergence of drug resistance (Bennett & Guyatt, 2000). An example of these monitoring procedures which could aid in the delay or prevention of drug resistance emergence would be to treat only a proportion of the people in an infected community at most risk (e.g. targeting school children). This would ensure that some wild-type nematodes remain in the community and the genes of these survivors would dilute those of nematodes experiencing selection pressure. Other procedures could include giving treatment at intervals greater than the nematodes generation time or changing the drug of choice for a particular control programme (De Silva *et al*, 1997). An established protocol for detection of suspected drug resistance would also be very useful.

Regarding other drugs such as the antischistosomals, no evidence of drug resistance to metrifonate has so far been reported. However, with oxamniquine and hycanthone, drug reistance has been located both in the field and the laboratory since the 1970's (De Silva *et al*, 1997). With Praziquantel, two recent developments have raised concerns. One report described low cure rates in infected patients in Senegal (Stelma *et al*, 1995), while another showed diminished susceptibility to praziquantel in a schistosome isolate from the same area in Senegal (Fallon *et al* 1995).

The threat of drug resistance emerging is therefore ever-present and development of new anthelmintics to take the pressure off the main-stream anthelmintics is therefore a necessity.

1.10 The need for new anthelmintics.

Effective agents are needed against systemic infections that respond inadequately to current drugs e.g. the filariases, echinococcosis, fascioliasis, dracunculiasis, trichinosis, toxocariasis and cysticercosis. The discovery of agents effective against all developmental stages of parasitic helminths, e.g. adult filarial worms, would constitute a major advance (Hardman & Limbird, 1996).

Unfortunately, owing to the majority of helminth infections being non-life threatening there is sometimes a lack of interest in research to develop new drugs, with more money being put into the development of drugs for more life threatening disease (Geary, 1999). The development however, of new anthelmintic drugs is highly important. This fact arises not only with the increasing possibility of resistance occurring within the human population due to the usage of the same anthelmintics for many years, but also when viewing the extremely high figures of helminth infection present in the world. This situation is highlighted in the chemotherapy of filarial infections which is currently not satisfactory. There is an urgent need for a new macrofilaricidal drug with only slight side-effects (Harder, 2002). Presently the only way is the prophylactic control of *Onchocerca volvulus* infections and treating lymphatic filariasis with ivermectin, DEC, ivermectin / DEC or ivermectin/albendazole combinations (Harder, 2002). Helminth infection, can cause much suffering in the human population with diseases such as trichuriasis causing stunting and poor mental development due to malnutrition (Bell, 1995). Helminth infections also cost the veterinary and food industries many millions of US dollars each year, and these facts stress the importance in the development of new anthelmintics.

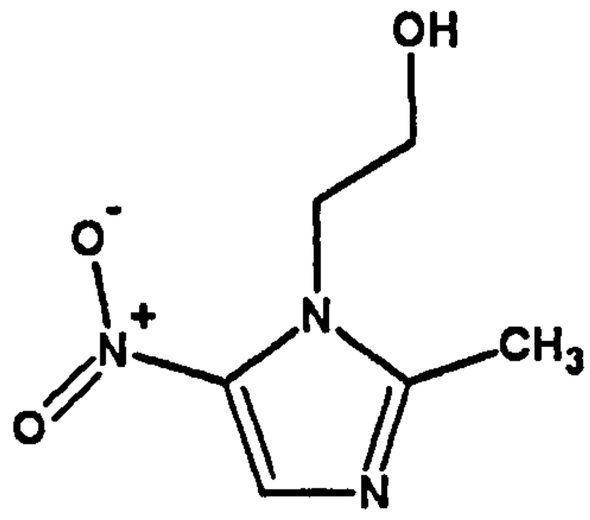
Recently the pharmaceutical industry has started to switch to mechanism-based, ultra high-throughput screening (UHTS) in the discovery of new drugs. Current understanding of the biochemistry of parasitic helminths and of the mechanisms of action of known anthelminthics identifies several targets that could be formatted for UHTS (Thompson *et al*, 1996). However, knowledge of helminth biology is presently very poor. Thus future investment in helminth biology is a necessity, as well as is the continuing development and understanding of novel compounds to combat worm infestation .

Efforts in this direction, have seen the emergence of a new anthelmintic, nitazoxanide (Stettler *et al*, 2003; Juan *et al*, 2002; Rossignol *et al*, 1998). This 5 nitrothiazole was originally designed and tested for toxicity towards anaerobic/microaerophilic protozoa and bacteria and was later found to have anthelmintic properties (Rossignol & Maisonneuve, 1984). The primary mechanism of nitazoxanide against protozoa and bacteria is thought to be similar to that of metronidazole. This idea was primarily circumstantial in that nitazoxanide affected a similar range of organisms to metronidazole and had a similar nitro-group.

1.11 Metronidazole.

Metronidazole [1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole] is the prototype for the nitroimidazole class of antimicrobials. Originally introduced over 25 years ago for the treatment of patients with *Trichomonas vaginalis* (Lamp, 1999) it has an extremely broad spectrum of activity against anaerobic and microaerophilic protozoa and bacteria which is used to clinical advantage. Metronidazole has particularly high activity *in vitro* and *in vivo* against the protozoa *Entamoeba histolytica* and *Giardia intestinalis* (Upcroft *et al*, 1999) as well as the bacteria *Clostridium* and *Helicobacter* sp (Hardman & Limbird, 1996). Sensitive isolates of *T. vaginalis* are killed on exposure to <0.05μg/ml of metronidazole under anaerobic conditions (Hardman & Limbird, 1996). Metronidazole has also been reported to produce a 60 % cure rate for *Fasciolopsis buski,* the largest trematode to infect man (Shah *et al*, 1973).

Figure 1.10: Structure of metronidazole.



1.11.1 Mechanism of action of metronidazole.

Metronidazole is reductively activated within microbial cells (Samuelson, 1999). Metronidazole has a low midpoint redox potential (Eo') of – 486mV (Wardman, 1985) and is selectively toxic for organisms capable of reducing its nitro group intracellularly to a toxic nitro free radical. This free radical would initially be a nitro anion and be produced by a one-electron reduction of the nitro group.

$$R\text{-}NO_2 + e^- \longrightarrow R\text{-}NO_2^{-\bullet} \qquad \text{EQUATION 1}$$

The reduction of metronidazole in *Helicobacter pylori* is carried out by an oxygen-insensitive nitroreductase (Goodwin *et al*, 1998) and in *Giardia intestinalis* and *Trichomonas vaginalis* by pyruvate ferredoxin oxidoreductase (Upcroft et al, 1999).

Inside the microorganism, the nitro radical, in the presence of minute concentrations of oxygen produces superoxide (Smith *et al*, 1995). The toxic effect inside the micro-organism is as follows:-

$$R\text{-}NO_2^{-\bullet} + O_2 \longrightarrow R\text{-}NO_2 + 0_2^{-\bullet} \qquad \text{EQUATION 2}$$

In this process the parent drug is regenerated and this had led to the term "futile redox cycling". Hydrogen peroxide is then formed by dismutation of 2 superoxide radicals into oxygen and hydrogen peroxide by superoxide dismutase (SOD).

$$2O_2^{-\bullet} + 2H^+ \xrightarrow{(SOD)} H_2O_2 + O_2 \qquad \text{EQUATION 3}$$

Superoxide can stimulate the production of the highly toxic hydroxyl radical $OH^{\bullet}$ by reducing ferric or cupric ions to ferrous or cuprous ions (a) which then undergo the Fenton Haber-Weiss reaction with hydrogen peroxide (b). In this diffusion controlled

reaction the production of the very toxic hydroxyl radical is shown in the following equation (Halliwell & Gutteridge, 1999).

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \qquad \textbf{(a)}$$

$$Fe^{2+} \text{ complex} + H_2O_2 = OH^{\bullet} + OH^{-} + Fe^{3} \text{ complex.} \qquad \textbf{(b)}$$

The hydrogen peroxide and oxygen products from equation 3 are broken down by the detoxification enzyme catalase to oxygen and water.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad \text{EQUATION 4}$$

The highly reactive, short lived hydroxyl radical is likely to be a major toxic product from nitroimidazole drugs like metronidazole. It can cause major damage to the synthetic machinery and DNA of the microorganism. 2-electron reduction, 3-electron reduction and 4-electron reduction of metronidazole also occur, which lead to the production of mutagenic electrophiles like hydroxylamines.

e.g. $R\text{-}NO_2 \xrightarrow{1e^-} R\text{-}NO_2^{\bullet -}$ (NITRO RADICAL) $\xrightarrow{1e^-} R\text{-}NO \xrightarrow{2e^-,\ H^+} R\text{-}NHOH$ (HYDROXYLAMINE) $\xrightarrow{2e^-,\ H^+} R\text{-}NH_2$ (AMINE)

1.11.2. Metronidazole resistance.

Resistance to nitroimidazoles is rare despite extensive use worldwide (Edwards, 1993) The first report of resistance was seen in *T. vaginalis* about two years after metronidazole was first introduced (Robinson, 1962). Resistance has also been reported in *Bacteroides fragilis* (Chardon, 1977) and *Mobiluncus* spp (Spiegal, 1987, Sprott, 1983). In *Trichomonas* spp, resistance to metronidazole and other 5- nitroimidazoles is characterised by a tolerance to oxygen resulting in a decreased susceptibility under both anaerobic and aerobic conditions (Edwards, 1993), which suggests that the organism becomes more resistant to oxygen in the environment.

1.11.3 Metronidazole versus Nitazoxanide.

Though metronidazole is active in anaerobes and microaerophiles, the activity of the 5-nitrothiazoles, nitazoxanide and its metabolite tizoxanide is rather wider. The possible reason for this useful property is that 5-nitrothiazole moiety of nitazoxanide (NTZ) (Eo' –estimated at 350mV) and tizoxanide (TIZ), is reduced by a wider range of anaerobic and microaerophilic organisms than metronidazole, leading to a broader spectrum of activity (D. C. Warhurst & D. J. Meyer (2000), unpublished data). This redox potential of nitazoxanide and tizoxanide is similar to that of another 5-nitrothiazole, niridazole (Eo' of – 390mV) (Wardman, 1985; Bassily *et al*, 1979) which also has a wide range of activity against parasites, however while niridazole is a mutagen, NTZ and TIZ are not (Romark Laboratories (1999) "Pharmaco-Toxicological expert report). NTZ is effective against *Helicobacter pylori* (Megraud *et al*, 1998) (which can cause peptic ulcer and lymphomas). *H. pylori* is able to reduce metronidazole because it has an oxygen-insensitive nitroreductase *rdxA* (Sisson *et al*, 2002). However, *H. pylori* possesses a PFOR which is incapable of reducing metronidazole and resistance to this drug is due to a defect in *rdxA* which renders the nitroreductase enzyme inactive and therefore stops reduction of metronidazole. However, strains resistant to metronidazole retain sensitivity to nitazoxanide (Sisson et al, 2002). This lack of reduction of metronidazole when the *rdxA* system is inactive is due to the redox potential of metronidazole being too negative and hence it cannot be reduced by *H.pylori* PFOR. The redox potential of nitazoxanide however is less negative and it therefore becomes likely that the *H. pylori* PFOR may be involved in the reduction of nitazoxanide (Sisson *et al*, 2002). This easier reduction of nitazoxanide and tizoxanide compared with metronidazole is supported by a comparison of calculated heats of formation of the reduced drugs, where the difference involved was estimated as 13 kcal/mol. When comparing niridazole and tizoxanide, this was estimated to be 3 kcal/mol (D.C.Warhurst 2000, unpublished). Furthermore, another differing factor of nitazoxanide and metronidazole is that the reduction products from nitazoxanide, although equally toxic for parasites, are not so indiscriminately mutagenic as those from metronidazole. The test for mutagenicity was carried out using the Ames test. This test using *Salmonella typhimurium* showed that nitazoxanide did not cause mutagenesis as seen with metronidazole (Romark, Pharmaco-Toxicological report, 1999; Sisson *et al*, 2002).

1.12 Nitazoxanide and its derivatives.

Nitazoxanide (NTZ) is 2-acetyloxy-*N*-(5-nitrothiazol-2-yl) benzamide and was first described in 1975 by Rossignol & Cavier. Derivatives of nitazoxanide include the metabolite tizoxanide (TIZ), denitro-nitazoxanide (DNNTZ), denitro-tizoxanide (DNTIZ), the excretory metabolite tizoxanide glucuronide (TIZg) and 2-benzamido-5-nitrothiazole (BZNT). Experimentally DNNTZ and DNTIZ are used to investigate the role of the nitro group.

* For structures of DNNTZ, DNTIZ and BZNT see Appendix III

Figure 1.11: Structures of nitazoxanide, tizoxanide and tizoxanide glucuronide.

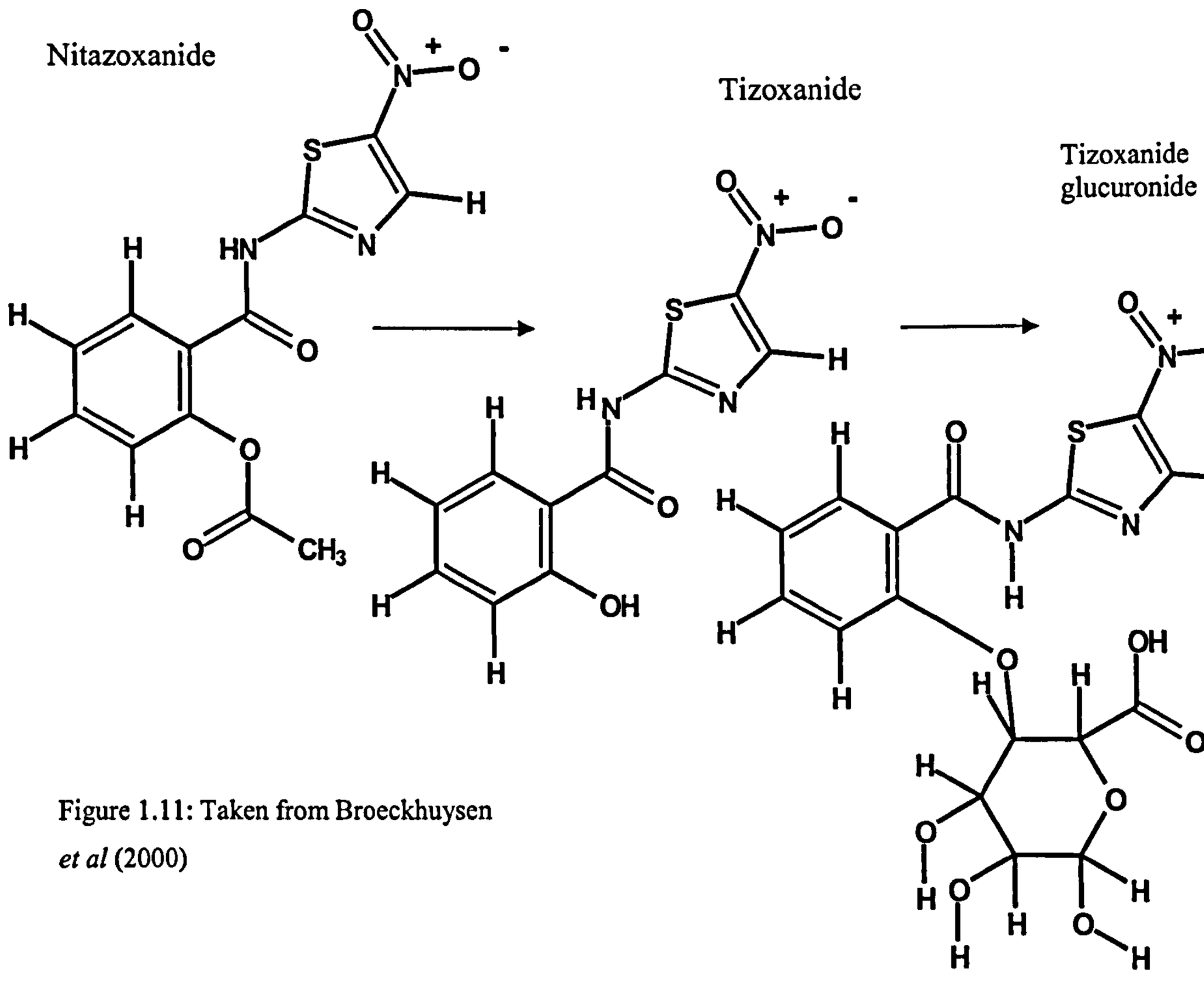


Figure 1.11: Taken from Broeckhuysen *et al* (2000)

Nitazoxanide is metabolised rapidly to tizoxanide, which is itself a potent antibacterial and antiparasitic agent (Dubriel *et al*, 1996). Further metabolism of tizoxanide leads to the formation of the glucuronide conjugate (Rossignol & Stachulski, 1999).

1.12.1 Pharmacokinetics.

Nitazoxanide is only partially absorbed from the gastro-intestinal tract, and 65% of the metabolised drug is excreted in the faeces (Broeckhuysen *et al,* 2000). At least 32 % of a 500mg oral dose is absorbed, much of which is secreted as the metabolite, tizoxanide glucuronide, in the bile. (see fig 1.12). Upon absorption, the acetyloxy group of nitazoxanide is rapidly hydrolysed to its first metabolite, tizoxanide, which is highly active against the same range of organisms as nitazoxanide (see figure 1.11). The final metabolite, a glucuronide conjugate (tizoxanide glucuronide), is less active, and is found in the plasma and urine and at high concentrations in the bile (Broeckhuysen *et al,* 2000). No trace of nitazoxanide can be found in either plasma or urine after the single dose oral administration (Stockis *et al,* 1996). This pattern of absorption shows that nitazoxanide could have antiparasitic effect both systemically and locally throughout the gastro-intestinal tract.

The metabolism of nitazoxanide has been investigated *in vitro*, in laboratory animals and in man, by studying the fate of radio-labelled drug and by cold analytical techniques including mass spectrometry and HPLC (Broeckhuysen *et al,* 2000). The de- acetylation occurs spontaneously over time in aqueous solution and is pH and temperature dependent, with more rapid de- acetylation at lower pH and higher temperature. In 1M HCl, 50 % of NTZ hydrolyzes to TIZ in 30 minutes and complete hydrolysis is observed after 8 hours. At 0.1M HCl (pH 1- stomach) less than 10% of NTZ hydrolyzes to TIZ. It is therefore not likely that NTZ hydrolyzes significantly in the stomach due to acid (D.J.Meyer 2000, unpublished data). The de-acetylation, probably occurs much more rapidly by the action of simple esterase enzymes, which are widely distributed in many mammalian tissues and intestinal secretions, as well in the microorganisms of the gut flora. The enzymatic de-acetylation is a very rapid process which probably accounts for the absence of detectable intact nitazoxanide from plasma and excreta (Romark, Pharmaco-Toxicological report, 1999; Stockis *et al*, 1996).

Figure 1.12: Absorption of nitazoxanide.

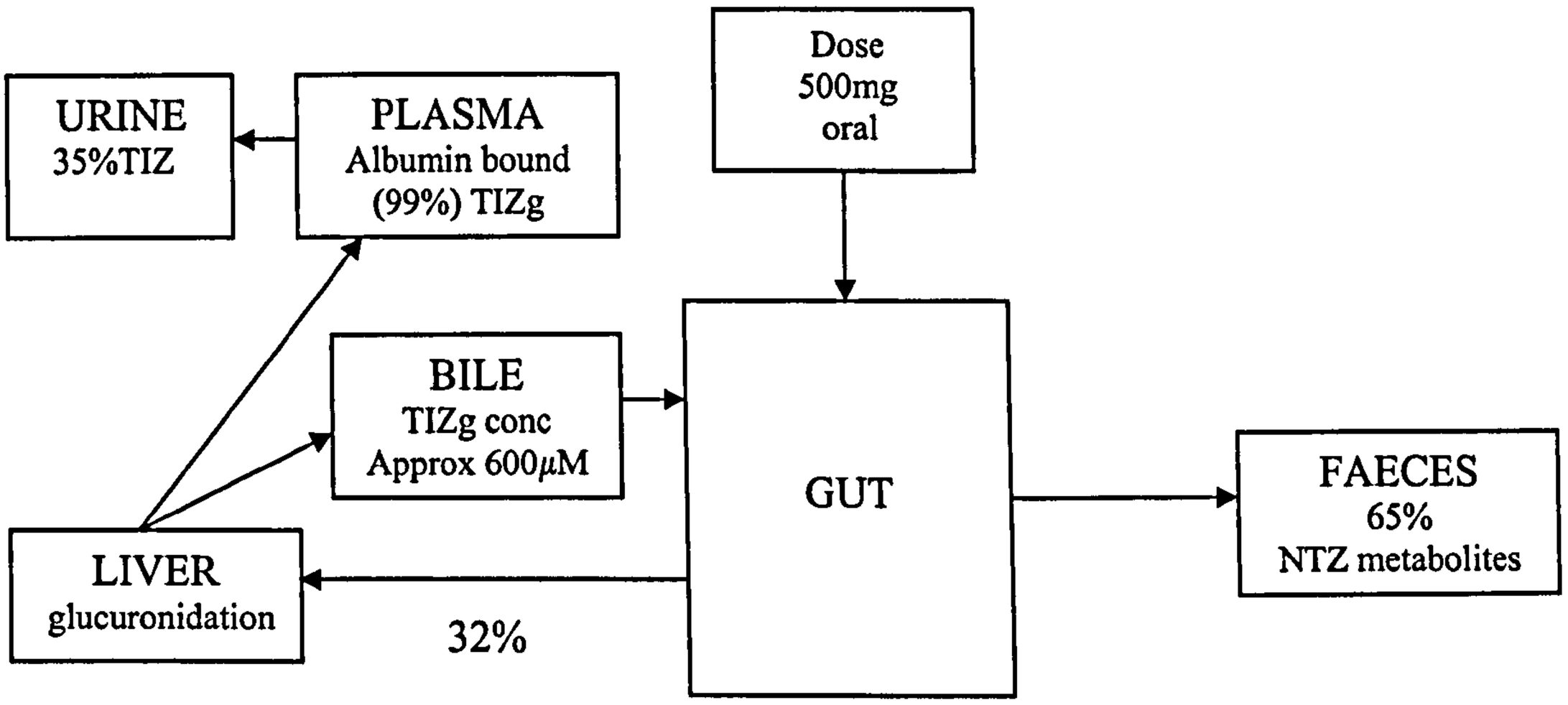


Fig 1.12: After absorption of a 500mg dose of nitazoxanide, the drug rapidly hydrolyses in the body to form its primary metabolite tizoxanide, which has a reactive hydroxyl group. This hydroxyl group facilitates glucuronidation and excretion in the urine and faeces (Broekhuysen et al, 2000). The fact that tizoxanide can form a glucuronide may account for the lack of significant toxicity to the host and explains why the drug is best suited for activity in the intestinal tract.

1.12.2 Physiochemical properties.

X-ray study of yellow crystals of tizoxanide carried out by Dr. John Lisgarten and Prof Rex Palmer of Birkbeck College London (unpublished observation), shows that the two rings are completely coplanar, and linked by an H-bond between >N-H and the phenolic –OH, which effectively gives a three ring structure (see Appendix III). At pH 4.0 solutions of tizoxanide are colourless with an absorbance peak of approximately 350nm. At pH 7.0 the tizoxanide solution is yellow with a peak absorbance of approximately 409nm. This indicates that the yellow species is the anion. At pH 4.0 tizoxanide is non-planar. At pH 7.0 however tizoxanide loses a proton and becomes a planar anion. Since the pKa of NTZ and TIZ is slightly acidic at 5.9 and 5.25 (see table 1.3) respectively, both are proton donors at around pH 5.0. One interesting characteristic of NTZ/TIZ is

their high plasma protein binding and studies using ultrafiltration found less than 1% NTZ and TIZ unbound in plasma (Romark laboratories investigation brochure, 1999). This would cause a significant depression of NTZ/TIZ availability in the blood stream.

NTZ at excitation 350nm, emission 480nm shows a high relative fluorescence (91 RFU) compared to TIZ and TIZg (see table 1.3). This fluorescence is lowered when the wavelength is increased to 414nm, emission 500nm. DNNTZ and DNTIZ demonstrate very high fluorescence at 350nm, emission 480nm, which is significantly decreased with an increase in wavelength.

Table 1.3 Physiochemical properties of NTZ and related drugs (D.J.Meyer, unpublished, 2000).

	NTZ	TIZ	TIZg	DenitroNTZ	DenitroTIZ
pKa[1]	5.9	5.25	7.1	6.1; 9.1	6.0; 7.3
P[2]	18.0	19.6			
E_o'(mV)[3]	~ -350	~ -350	~ -350		
Fluorescence[4]	91	44	51	167	5400
Fluorescence[5]	38	10	10	13	14

[1] Measured by scanning spectrophotometry.
[2] Partition coefficient between phosphate- buffered saline (pH 7.0) and 1- octanol at 37°C.
[3] Estimated using spinach NADPH ferredoxin reductase and ferredoxin to reduce various nitro- compounds (including those of known E_o') and following oxygen uptake (hence superoxide formation) in a 0.5ml Rank oxygen- electrode cell.
[4] Relative fluorescence units using Spectramax Gemini plate-reader to obtain optimal wavelengths, then make fixed- wavelength readings: excitation 350nm, emission 480nm.
[5] As above, excitation 414nm, emission 500nm.

NTZ and TIZ are weakly lipophilic with calculated log P (P= distribution ratio between octanol and water at equilibrium) values of 1.8 and 2.02 respectively (see table 1.4). These log P values are high enough to allow absorption through the cuticle of a nematode, though levamisole and ivermectin which have higher log P values would be more readily able to cross nematode cuticles. The lower log P value of NTZ and TIZ suggests that any possible drug effect on nematodes would be delayed compared with that of LEV and ivermectin. In order for NTZ and TIZ to be

protonophores, they would need to be readily lipophilic to dissolve in the mitochondrial membrane and have a pKa around 5.0.

Table 1.4 Lipophilic values (Log P) and other features of anthelmintic drugs.

	Log P (calc)	Log P measured	Proton acceptor	Proton donor
Nitazoxanide	1.8	1.67	7	1
Tizoxanide	2.02	2.10	5	2
Mebendazole	2.52	2.71	5	2
Praziquantel	2.16		4	0
Ivermectin A	5.60		10	2
Levamisole	2.79	2.87	2	0
Niclosamide	3.51	4.56	4	2
CCCP	3.15	3.38	1	1

A high number proton acceptor shows that the compound is more hydrophilic.

1.13 Clinical usage.

In the United States nitazoxanide has been used as an investigational new drug for the treatment of diarrhoea caused by *Cryptosporidium parvum* and microsporidia in patients with AIDS. The anticryptosporidial activity of NTZ was first reported by Doumbo *et al*, in 1997. The results of a double blind, placebo- controlled study of NTZ in 66 Mexican AIDS patients with cryptosporidiosis demonstrated the efficacy of the drug. A 500mg dose twice a day for 14 consecutive days or a 100mg dose twice a day for 14 consecutive days had a cure rate of 63% and 67% respectively. These groups differed statistically compared to placebo with P values of 0.016 and 0.013 respectively. Diarrhoea was also resolved in 90% of the patients who exhibited eradication of the cryptosporidium infection in this trial. (Rossignol *et al*, 1998). Soave, 2002 reported the results of a study carried out in the United States, in 30 AIDS patients with cryptosporidial diarrhoea who did not respond to other potential treatments. Ten of the twelve with CD4 count below 54cells/mm^3 (83%) who completed at least 12 weeks of treatment with NTZ eradicated the parasite (Soave and Davis 2002). A double blind placebo controlled study of NTZ in 50 adults and 50 children in Egypt showed clinical and parasitological response of 80% and 67% respectively ($p<0.0001$ for the entire

population) and this was the first trial to study treatment of cryptosporidiosis in immunocompetent individuals (Rossignol *et al*, 2001). However recent clinical trials in Zambia have shown treatment with NTZ produces cure rates of 52% of *Cryptosporidium* in HIV- seronegative children, whereas HIV-seropositive children did not benefit from NTZ treatment. (Amadi et al, 2002).

In November 2002, nitazoxanide was Romark's first product to reach the market in the United States and is marketed as Alinia™. Alinia was approved by the Food and Drug Administration (FDA) as an oral suspension for the treatment of diarrhoea caused by *Cryptosporidium parvum* or *Giardia intestinalis* in children from 12 months to 11 years of age. (Romark Labs, 2001). Romark is currently pursuing FDA approval to market Alinia tablets in the United States for the treatment of diarrhoea caused by *Cryptosporidium parvum* and *Giardia intestinalis,* including *Cryptosporidium*-induced diarrhoea in patients with AIDS.

In Latin America nitazoxanide 500mg film-coated tablets, 200mg dispersible tablets and a 100mg/5ml paediatric suspension are marketed. These are used for the treatment of a broad spectrum of parasitic diseases that are common in the developing world and also for treatment of diarrhoea caused by *C. parvum* and microsporidia in AIDS patients (Romark Labs, 2001).

1.14 NTZ activity against anaerobic protozoa and microaerophilic bacteria.

Nitazoxanide has activity against the organisms described earlier for metronidazole e.g. *Giardia intestinalis*, *Entamoeba histolytica,* and the *Bacteroides fragilis* group (Dubreuil *et al*, 1996). Nitazoxanide also kills other species including *Propionibacterium* spp, and *Bifidobacterium* spp (Dubreuil, 1996), *Clostridium difficile* (McVay, 2000) *Cryptosporidium parvum* (Gargala *et al,* 2000), *Blastocystis hominis* and *Isospora belli* (Romero Cabello, 1997).

In vitro nitazoxanide and tizoxanide have shown very good activity against *E. histolytica* (strain HM1) with concentrations of 0.06 μg/ml* inhibiting 100% growth of the parasite. This compares favourably to metronidazole, which only gave 100%

inhibition at 0.12µg/ml (Adagu *et al*, 2002). With *G. intestinalis* tizoxanide was 8 times as active as MTZ and is twice as active as MTZ in a resistant strain. Nitazoxanide and tizoxanide have also been demonstrated to be effective against 16 metronidazole-resistant clinical isolates of *T. vaginalis* cultured in aerobic and anaerobic conditions. In aerobic conditions all strains were resistant to metronidazole with MLC_{50} of 100µg/ml. NTZ and TIZ were effective against all strains with MLC_{50s} of 1.6 and 0.8µg/ml respectively. In anaerobic conditions all strains had decreased sensitivity to metronidazole with a MLC_{50} of 12.5µg/ml. NTZ and TIZ were effective with MLC_{50}'s of 1.6 and 0.4µg/ml respectively (Adagu *et al*, 2002).

(* For conversions of µg/ml to µM for NTZ and related compounds, see Appendix IV).

One important finding with regards to nitazoxanide is its activity against *C. parvum. C. parvum* was cultured *in vitro* using the MDBK cell line, selectively cloned to make them susceptible to the parasite. After 48 hours of incubation the cultures were read using an immuno-fluorescent technique, and it was seen that nitazoxanide reduced the percentage of cells infected compared to the controls by 93% and 44% for concentrations of 10µg/ml and 1µg/ml respectively (Theodos *et al*, 1998). *In vivo* nitazoxanide reduced the number of oocysts in ICR female mice by 57.7% and 95.7% for 100 and 150mg/kg respectively when compared to the control animals (Romark Pharmaco Toxicological Report, 1999). This activity against *C. parvum*, shows the benefits of nitazoxanide over metronidazole which has no effect against this parasite.

1.15 Nitazoxanide and helminths.

Nitazoxanide has a broad action against helminths and in clinical trials, it has demonstrated promising cure rates (Davila-Gutierrez *et al*, 2002). A 3-day treatment course was reported to be effective in treating helminth infections caused by *Ascaris lumbricoides,* hookworms, *Trichuris trichiura, Taenia saginata,* and *Hymenolepis nana* (Rossignol *et al*, 2001; Davila-Gutierrez *et al*, 2002) (see table 1.5). The fact that nitazoxanide affects the same anaerobic species as metronidazole as well as having activity against helminths suggests that it may have a similar mode of action but with important differences.

Recent clinical trials of a standard 3-day course of NTZ against helminths have shown cure rates comparable to the standard single 400mg dose of albendazole in the treatment of ascariasis, to praziquantel (25mg/kg) in the treatment of hymenolepiasis and a higher cure rate than a single 400 mg dose of albendazole in the treatment of trichuriasis (Ortiz *et al*, 2002). Nitazoxanide compares well with praziquantel and albendazole, with all three drugs producing egg reduction rates in excess of 98% in ascariasis, trichuriasis and hymenolepiasis (Juan *et al*, 2002).

Table 1.5 Efficacy of nitazoxanide against helminths.

Species/level of infection	Dose	% Cure
A. lumbricoides (Nematode) Light Moderate Heavy	45mg/kg dose divided into 6 doses over 3 day period (7.5mg/kg twice/day)	96% 82% 48%
T. trichiura (Nematode) Light Moderate	45mg/kg dose divided into 6 doses over 3 day period	80% 56%
Hookworms (Nematode)	As above	96%
Hymenolepis nana (Cestode) Light Moderate	As above	93% 84%
Taenia saginata (Cestode)	30mg/kg dose divided into 6 doses over 3 days	95%
F. hepatica (Trematode)	7.5mg/kg twice/day for 7 days	87%

(Unpublished Romark data, 1999)

Apart from a heavy infestation with *A. lumbricoides* (48% cure) or a moderate infection with *T. trichiura* (56% cure), the efficacy of nitazoxanide meets the World Health Organisation standard for an antiparasitic drug by having a cure rate above 60% (WHO, 1990). Cestodes are particularly susceptible with cure rates of 93% and 95% for *H. nana* and *T. saginata* respectively.

1.15.1 *In vitro* activity of NTZ against helminths.

With the trematode *Fasciola hepatica*, nitazoxanide at 32μM *in vitro* caused rapid contraction, ventral curling and eventually death (Romark laboratories & Malone, Final Report, 1995). Tizoxanide was as effective but over a longer period of time. At the lower concentration of 3μM, NTZ was still toxic, but death of the fluke could take up to 3 days.

In vitro studies using *Echinococcus multilocularis*, showed vacuolisation of the germinal layer of the metacestode, damage to the microtriches and separation of the laminated and germinal layer when exposed to 32μM NTZ (Stettler *et al,* 2003).

1.16 Toxicity of NTZ.

At therapeutic doses, NTZ has low toxicity when given orally. Toxic manifestations at high doses include loose stools, emesis (observed in dogs) and anaemia (Romark, Pharmaco-Toxicological report, 1999). Studies carried out by Romark (unpublished, data) show that NTZ is not teratogenic and does not affect fertility nor impair peri- and post-natal development.

1.17 Possible Mechanism of action of NTZ.

Although it is almost two decades since the introduction of nitazoxanide, it has only been recent work which has demonstrated the broad- spectrum anti-parasitic properties of the drug. The mode of action of nitazoxanide and its derivatives is as yet not completely known but preliminary tests have shown a number of possibilities. One

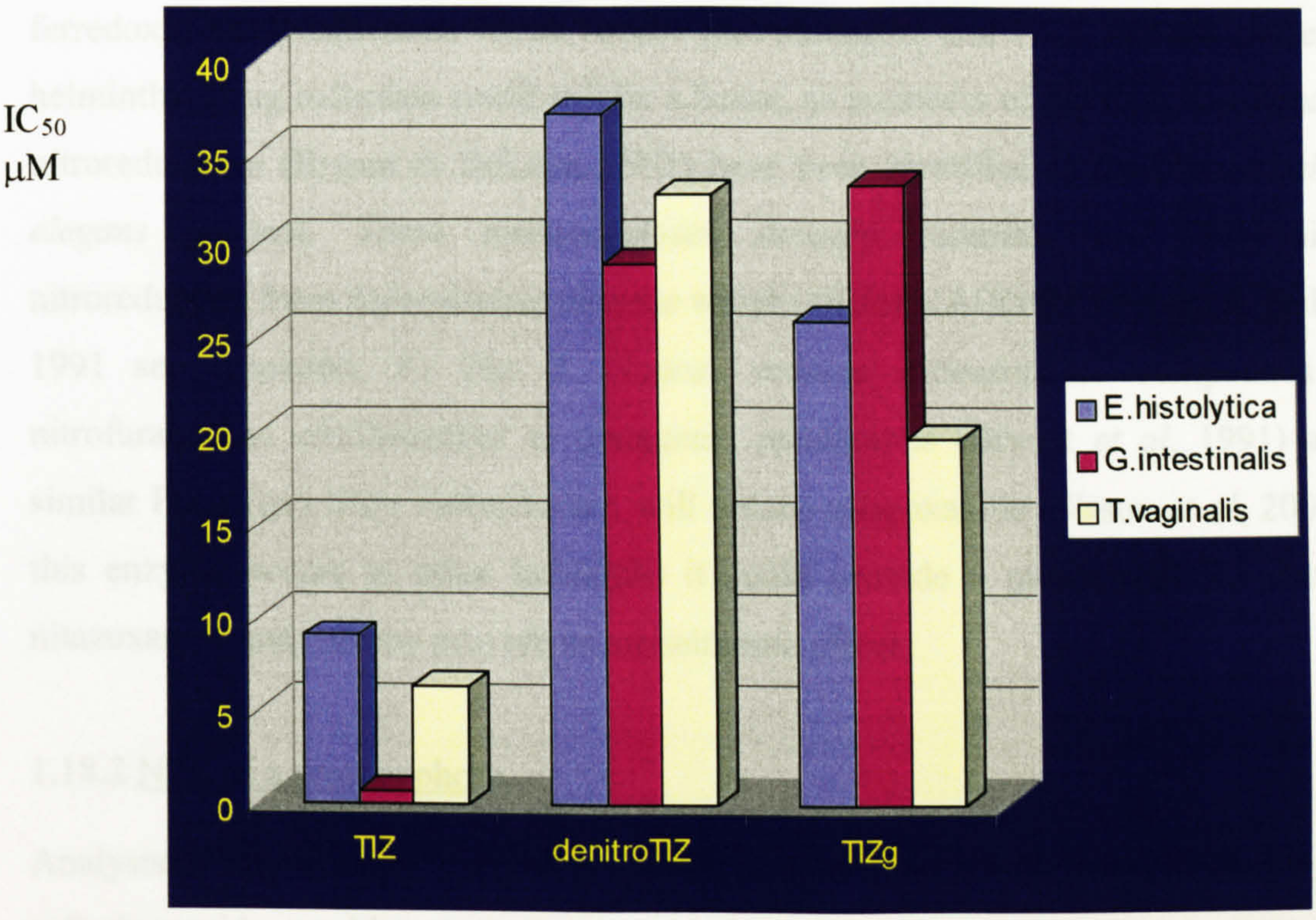
possibility, is that nitazoxanide undergoes a one electron reduction in a similar way to metronidazole. This has been demonstrated using spinach NADPH ferredoxin reductase (D. J. Meyer 2000, Unpublished data). Another possibility is that nitazoxanide acts as a protonophore (D. C. Warhurst 2000, Unpublished data).

1.17.1 Possible mechanism of action of NTZ against anaerobic protozoa/ microaerophilic bacteria: Effect of the nitro group.

As described earlier (section 1.11), the reduction of the nitro-group of metronidazole yields very reactive metabolites and hence very good activity against anaerobic intestinal parasites. Nitazoxanide and tizoxanide (see Fig 1.11) also have a nitro-group, which could undergo one electron reduction and recycling like metronidazole to yield significant amounts of superoxide radical anion (D. J. Meyer 2000, unpublished data), and hence become toxic to the anaerobic protozoa and microaerophilic bacteria. Comparing NTZ/TIZ to DNTIZ, the latter shows very poor activity against *E. histolytica, G. intestinalis and T. vaginalis* suggesting that the nitro-group is essential for anti-protozoal activity (Adagu *et al*, 2002).

TIZg the glucuronide metabolite of TIZ has a weaker *in vitro* antiparasitic activity, possibly because of its reduced cellular uptake (see figure 1.13) (Adagu *et al*, 2002).

Figure 1.13. Comparison of inhibitory effects of TIZ, DNTIZ and TIZg on *E. histolytica, G.* and *T. vaginalis.*



Data from Adagu et al, 2002

Figure 1.13: TIZ shows good activity against *E. histolytica, G. intestinalis* and *T. vaginalis*. DNNTZ and TIZg show a much weaker activity.

1.18 Possible mechanism of action of NTZ against helminths.

Nitazoxanide is active against nematodes, cestodes and trematodes. The mechanism of its anthelmintic activity is not fully understood, but there are a number of possibilities. While the nitro-reducing capabilities of parasites are an essential requirement for its action against anaerobic protozoa, this does not aid the explanation of the mechanism of NTZ in helminths, which are incapable of reducing metronidazole.

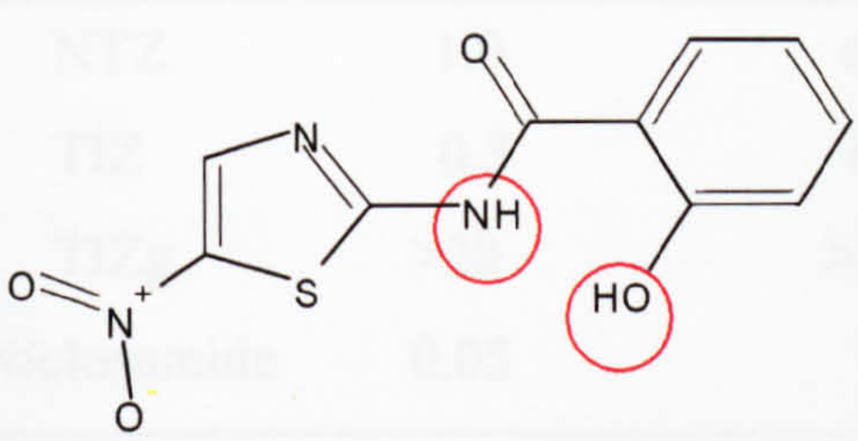
1.18.1 Drug reduction in helminths.

Nitazoxanide as stated is thought to work against protozoa by reduction of the nitro-group and hence produce a free radical toxic to the parasite. However, the pyruvate ferredoxin oxidoreductases which reduce metronidazole and NTZ are not present in helminths. Drug reduction could still be a factor, as members of the oxygen-insensitive nitroreductases (Bryant & DeLuca, 1991) have been identified in the *Caenorhabditis elegans* genome. These nitroreductases strongly resemble the FMN linked nitroreductase from *Enterobacter cloacae* which utilises NAD(P)H. (Bryant & DeLuca, 1991 see Appendix V) The *E. cloacae* reduces nitroaromatic compounds e.g. nitrofurans and nitrobenzenes to mutagenic metabolites (Bryant *et al*, 1991) and a similar FrxA (qv) from *Helicobacter,* will reduce nitazoxanide (Sisson *et al*, 2002). If this enzyme occurs in other helminths it could provide a mechanism for reducing nitazoxanide and thereby provide an anthelmintic effect.

1.18.2 NTZ as a protonophore.

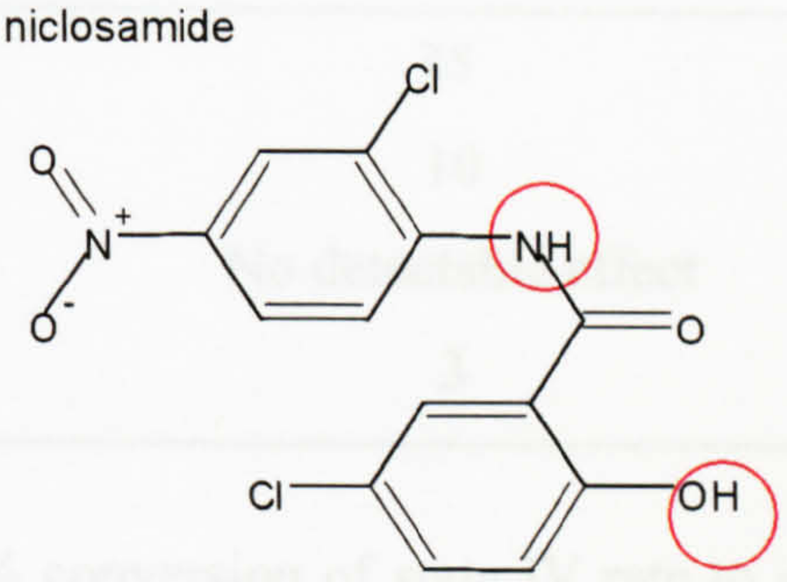
Analysis of the structure, spectral properties and partition coefficients of (nitazoxanide and) tizoxanide would suggest the drug could be a protonophore. Both an NH group and OH group are present (see fig 1.14) in a relationship seen in the known uncoupler niclosamide. The *pK*as of TIZ and NTZ and partition coefficients between PBS and octanol (see table 1.3) also suggest that NTZ and TIZ are uncouplers (D.J.Meyer 2000, unpublished). Both TIZ and NIC have a weakly acidic >NH group attached to an aromatic ring, The nitro group withdraws electrons from >NH which reduces proton attraction and the p*K*a value of proton release to around 4-8. The more easily dissociated is the proton from >NH, the higher will be the uncoupling power. Another major feature of the molecule is lipophilicity. The higher the log P value, the higher will be the uncoupling power. This is because uncouplers need to carry H^+ through the mitochondrial inner membrane. TIZ and niclosamide have high log P values and the potential to donate protons, thus making them good uncouplers Structural comparison with levamisole suggest that this drug could not donate a proton, and although the log P value is high, levamisole would not have any uncoupling properties.

Figure 1.14. Structures of tizoxanide and niclosamide showing possible protonophoric groups.

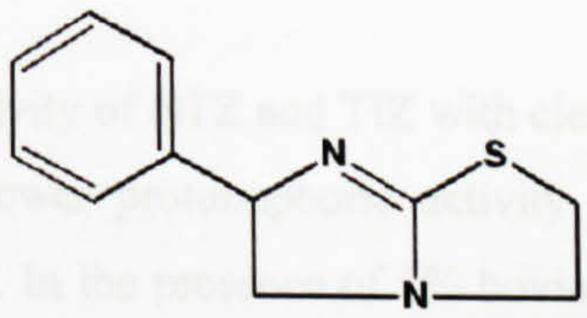


tizoxanide

niclosamide



levamisole



○ Possible protonophore groups.

The potential protonophoric activity of NTZ and TIZ were (hence) examined and confirmed by uncoupling respiration of cultivated human Caco-2 intestinal cells and proton-leakage in *Giardia* (table 1.6) (D. J. Meyer 2000, unpublished).

Table 1 .6 Table showing protonophoric action of NTZ, TIZ, TIZg and NIC on cultivated human intestinal Caco-2 cells.

Compound	U_{50}[1] (μM)	U_{50} (μM) With 1% BSA	Concentration (μM) causing 50% increased Caco-2 cell respiration.
NTZ	1.0	6.5	35
TIZ	0.3	6.5	10
TIZg	>20	>60	No detectable effect
Niclosamide	0.05	4	3

[1] concentration causing 50% uncoupling, i.e 50% conversion of state IV rate to state III rate of oxygen uptake in liver mitochondria.

Table 1.6: shows protonophoric activity of NTZ and TIZ with clear uncoupling evident with mouse liver mitochondria. TIZg shows much lower protonophoric activity and this agrees with the lower anti-protozoal potency of this compound. In the presence of 1% bovine serum albumin (BSA) the activity of both NTZ, TIZ and niclosamide was diminished showing that the binding of serum albumin to these drugs dramatically lowers their potency.

The role of protonophoric activity with regards to the anti-protozoal action of NTZ has previously been examined by comparison with the effects of other protonophores (D. J. Meyer 2000, unpublished). Growth of both *G. intestinalis* and *T. vaginalis* were sensitive to NTZ and TIZ as well as to protonophoric uncouplers lacking a nitro-group (CCCP or FCCP) (see table 1.7) showing that this property of NTZ/TIZ could contribute to its anti-protozoal activity. The protonophoric anthelmintic agent niclosamide also inhibited the growth of *T. vaginalis*. If an uncoupling effect of nitazoxanide could be demonstrated in helminths, it would suggest that NTZ/TIZ may have a similar mode of action as niclosamide.

Table 1.7 Inhibition of 'anaerobic' protozoal growth by protonophores[1]

Compound	*E.histolytica* HM1(s)	*G. intestinalis* EBE (s)	*T. vaginalis* ATCC50143 (r)
	I_{50} (μM)		
FCCP	-	12	1.3
CCCP	>80	-	1.4
Niclosamide	-	-	3
TIZ	11	2.3	3
MTZ[2]	18s 52r*	6s 15r *	3.9s* 59.6r

[1] I. S. Adagu, D. J. Meyer, unpublished.

[2] Metronidazole (MTZ) (not a protonophore) comparative values (Adagu *et al*, 2002);
s = MTZ- sensitive strain; r = MTZ- resistant strain.

* MTZ resistant strain of a) *E. histolytica* = HK9C2; b) *G. intestinalis* = JKM1.
MTZ sensitive strain of *T. vaginalis* = PER 013/EPM.

1.18.3 Potential neuromuscular activity of NTZ.

Selective neuromuscular toxicity against helminths may also be a mode of action of NTZ. Drugs like levamisole and pyrantel, utilise this mode of action against helminths. Both drugs are agonists for nicotinic acetylcholine receptors (nicotinic AchRs) (Martin et al, 1997) and after treatment, cause expulsion of the worm from the host. *In vivo* trials of nitazoxanide have demonstrated, that after drug treatment, the worm is expelled from the host (Romark 1999, unpublished data), an action consistent with a neuromuscular agent. This expulsion of parasites due to treatment with nitazoxanide could suggest a neuromuscular effect on the worm, similar to that of a known neuromuscular agonist. However expulsion can also result due to worm death or the worm ceasing movement.

1.18.4 Possible enzyme effect.

Another possible mode of action of nitazoxanide is the possible inhibition of essential parasite enzymes. Drugs like pyrantel, inhibit parasite acetylcholinesterase, causing an accumulation of acetylcholine leading to an excessive effect at the neurotransmitter. It is a possibility that NTZ action on the helminth may involve this mode of action.

The inhibition of fumarate reductase has also been proposed as a potential mechanism of NTZ by Kuramochi *et al*, 1995. Inhibition of this enzyme would inactivate a cycle within the helminth essential for anaerobic metabolism. Another possibility is (as mentioned) that helminths possess a nitroreductase type enzyme, which would be able to reduce the nitro-group of nitazoxanide in a similar mechanism to that of metronidazole i.e a drug-activation process by a parasite enzyme and not the inhibition of an enzyme.

1.18.5 Anti tubulin activity of NTZ.

Another mechanism of action proposed for NTZ is that NTZ could act like a benzimidazole drug and prevent ß-tubulin polymerising into microtubules. This mechanism has been investigated by D.J.Meyer using bovine brain tubulin (Sigma, UK). The results showed, as expected a significant inhibition of tubulin polymerisation by 34μM mebendazole (120% of control) and a good inhibition of tubulin by 83μM thiabendazole (58% of control). DMSO, 65μM NTZ and 57μM TIZg showed no tubulin inhibition. However, 38μM TIZ gave a slight reduction in bovine tubulin (79% of control), but this was found later to be due to the drugs insolubility at this concentration (D. J. Meyer 2000, unpublished data). This data would suggest that NTZ produced no inhibition of the bovine brain tubulin polymerisation. Further investigation is necessary as to confirm this observation.

1.19 Selection of helminths to study NTZ activity/mechanisms.

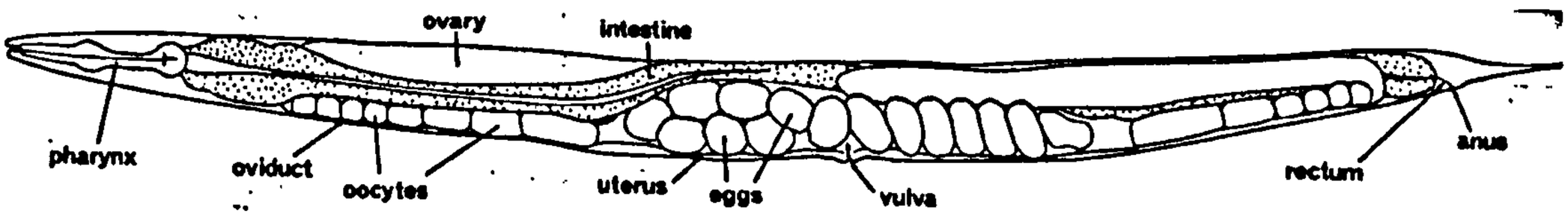
In order to study the anthelmintic drug mechanism of NTZ etc, the free-living *Caenorhabditis elegans* was chosen as a nematode model. Effects were compared to effects of known anthelmintics. Further investigation was then carried out to compare drug effects observed with *C.elegans* to those with parasitic nematodes, and with helminths from the Classes Cestoda and Trematoda.

1.19.1 *Caenorhabditis elegans.*

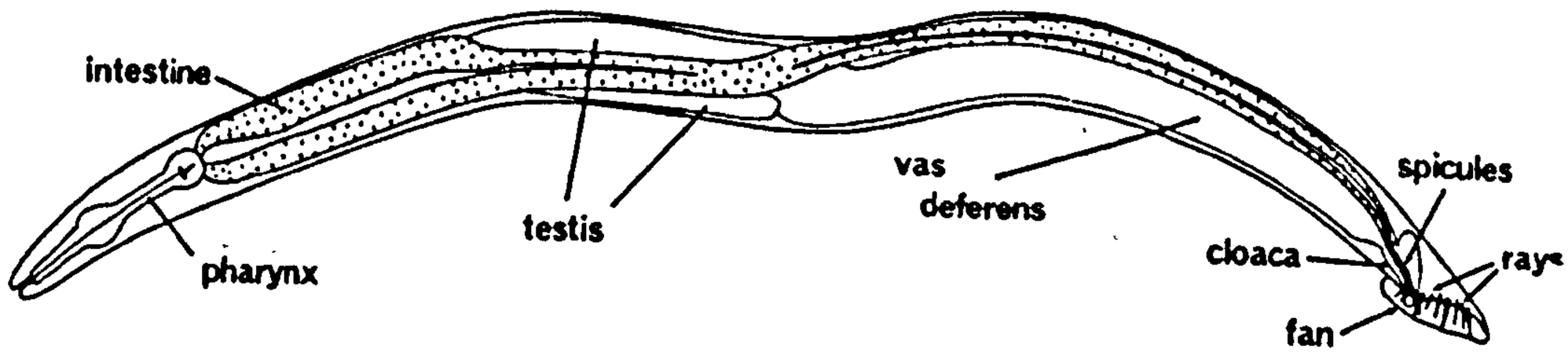
Caenorhabditis elegans is a small, free-living soil nematode that has been reported from many parts of the world (Wood 1988). It primarily feeds on bacteria and reproduces with a life cycle of about 3 days under optimal conditions, consisting of four larval stages (L_1-L_4) and the adult stage. There are two sexes of *C. elegans*, males and hermaphrodites. Each of these sexes, about 1mm in length as adults, differ slightly in appearance (see figure 1.15).

Figure 1.15 Major anatomical features of a) hermaphrodite and b) male *C.elegans*. (Wood, 1988).

a)



b)



Hermaphrodites produce both oocytes and sperm and can reproduce by self-fertilization. Males which are only found at a low frequency in the population ~ 0.2%, can fertilize hermaphrodites; hermaphrodites cannot fertilize each other.

A hermaphrodite that has not mated lays about 300 eggs during its reproductive life span (Wood, 1988). Juvenile worms hatch and develop through four larval stages (see figure 1.16), punctuated by moults with no metamorphosis. The mature adult emerging from the fourth moult is fertile for about 4 days and then lives for an additional 10-15 days. Upon starvation and overcrowding, an alternative third developmental stage, the *dauer* stage, accumulates in a population. Dauer larvae are arrested in development and can survive for several months (Broeks, 1997). In recent studies, this non-feeding dauer larvae stage has been shown to have an increased reliance on anaerobic respiration for energy production (Tartar & Rand, 2002).

Figure 1.16 Life cycle of C. *elegans*.

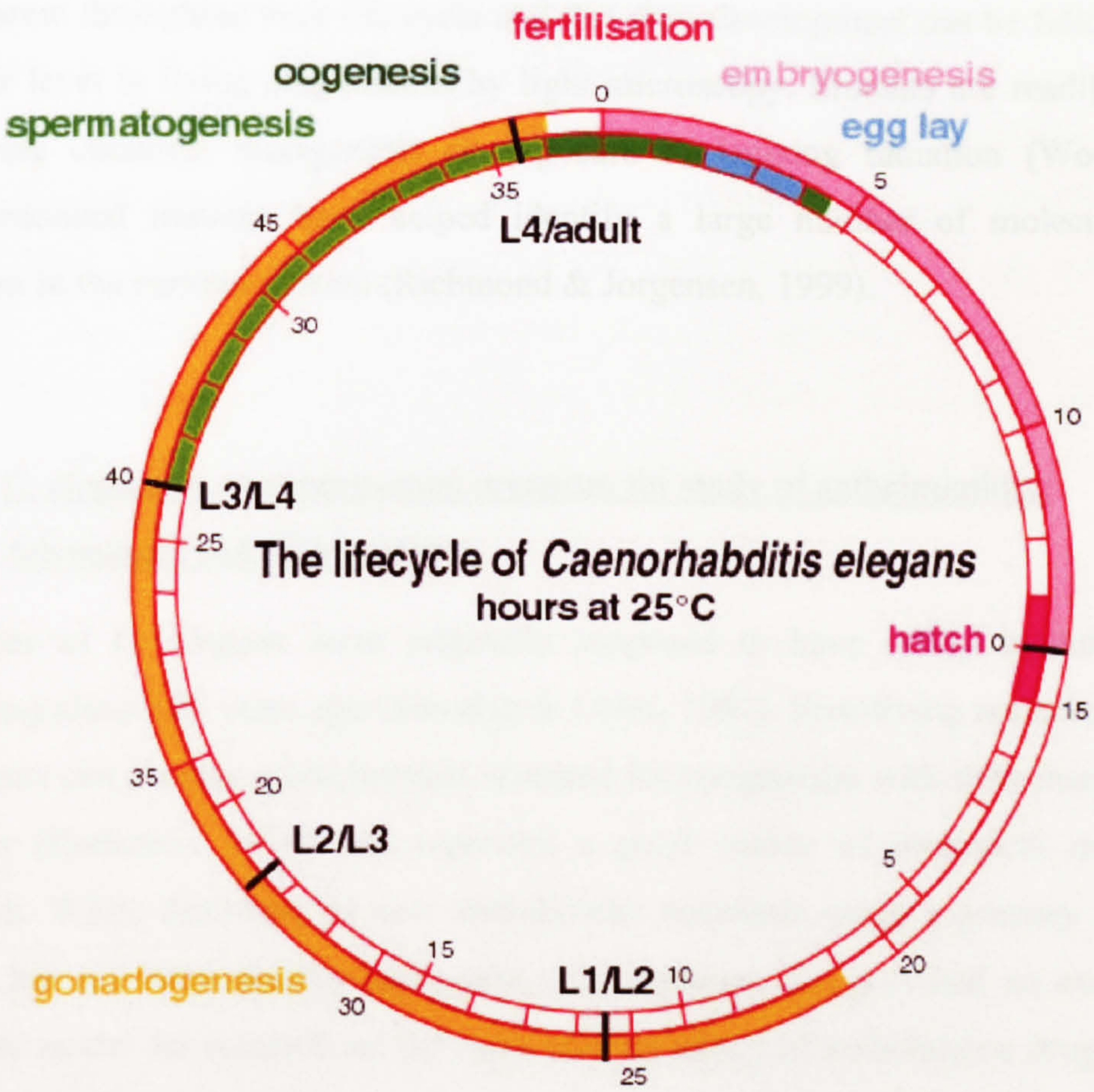


Figure 1.16 taken from http://ww2.mcgill.ca/biology/labs/roy/lifecycle.htm

Figure 1.16 represents the lifecycle of *C. elegans* at 25°C. As the temperature decreases, the length of each stage increases.

1.19.2 *C. elegans* as an organism for molecular research.

C. elegans has many qualities that are advantageous for molecular biological research (Broeks, 1997) It is a simple organism both anatomically and genetically (Wood, 1988) and has a very short life cycle (Burglin *et al*, 1998). The adult hermaphrodite has only 959 somatic nuclei, and the adult male has only 1031. The haploid genome size is $8x10^7$ nucleotide pairs, about eight times that of the yeast *Saccharomyces* or one-half that of the fruit fly *Drosophila* (*C.elegans* sequencing consortium, 1988).

C. elegans is easily maintained in the laboratory, where it can be grown on agar plates with *Escherichia coli* as food source, or axenically in complex liquid media. Individual animals are conveniently observed and manipulated with the aid of a dissecting microscope, and large numbers can be grown in mass culture. The use of *C. elegans* in an experimental drug assay is very convenient, owing to the fact that the worms are transparent throughout their life cycle and that their development can be followed at the cellular level in living preparations by light microscopy. Mutants are readily obtained following chemical mutagenesis or exposure to ionizing radiation (Wood, 1988). Uncoordinated mutants have helped identify a large number of molecules which function in the nervous system (Richmond & Jorgensen, 1999).

1.19.3 *C. elegans* as an experimental organism for study of anthelminthics: Advantages and disadvantages.

Cultures of *C. elegans* were originally proposed to have utility in anthelminthic screening almost 20 years ago (Simpkin & Coles, 1981). Free-living nematodes such as *C.elegans* can provide a biochemical standard for comparison with their many parasitic cousins (Rothstein, 1974) and represent a good source of biological material for research. While discovery of new anthelmintic templates using a primary *C. elegans* screen has not been notably successful, this organism has provided an exceptionally valuable model for research on the basic pharmacology of anthelmintic drugs (Rand & Johnson, 1995).

The use of free-living nematodes as experimental animals for biochemical investigations offers several distinct advantages (Rothstein, 1974). The most important of these is that the organisms can be grown in axenic media, which removes the presence of secondary organisms which could complicate experimental results.

It is moderately easy to detect drug effects in cultures of *C. elegans* by monitoring the behaviour, survival and/or reproduction of the worm. Drugs that reduce motility or survival, such as levamisole and the AM (avermectins and milbemycins) class, can be detected in these cultures at low concentrations, and their potency against *C. elegans* is a reasonable predictor of potency against parasitic nematodes in culture. Nicotinic receptor agonists have specific and easily assayed effects on several aspects of *C. elegans* behaviour including locomotion, feeding and egg-laying (Waggoner *et al*, 2000). However, *in vitro* testing is not comparable to *in vivo* results. Furthermore, the tetrahydropyrimidines, morantel and pyrantel, which act with similar potency to levamisole at parasitic nematode nicotinic receptors (Martin, 1997) are *in vitro* 50 to100 fold less potent respectively than levamisole against *C. elegans* (Simpkin and Coles, 1981). Differences *in vivo* between the tetrahydropyrimidines and levamisole are much less marked. In addition, the benzimidazoles (anti tubulin drugs) e.g. mebendazole, typically show low potency and slow onset of activity against *C. elegans* compared to their effect on parasitic helminths (Geary 1999). Finally, closantel, a salicylanilide similar to niclosamide with potent activity against *H. contortus in vivo* (Rothwell, & Sangster, 1993), is only weakly active against *C. elegans*.

Perhaps the most important disadvantage of the use of *C. elegans* as a model to estimate intrinsic potency of anthelmintics against parasitic nematodes are the large differences that are present in the life styles. The conditions that can be tolerated by a free-living nematode in culture compared to a parasite adapted to various host environments will in all likelihood show many differences. For example, a parasite in the host, will be affected in varying degrees to temperature, availability of nutrients, host immunity, threat of digestive enzymes etc, whereas a worm in culture will most likely be tested in ideal conditions. Subtle drug-induced alterations which result in expulsion of worms from a host, may be difficult to detect in culture. Geary, (1999)

points out that although the *in vitro* system can usually be manipulated to detect known anthelmintics, it is poorly suited to characterize the intrinsic potency of new compounds with unknown mechanisms of action.

1.19.4 *C. elegans* genome.

The *C. elegans* genome project was the first animal genome to be completed, around Christmas 1998. From the genome sequence, 19099 genes have been identified and novel features of gene organisation and chromosomal structure discovered (Blaxter, 2003). The cDNA data obtained from mRNA is used in the prediction of genes from the genome sequence along with database searches on the genomic sequence for similarities to genes of other organisms (such as parasitic worms and even humans) (Blaxter, 2003). *C. elegans* has proved to be an invaluable tool for the understanding of vertebrate neuronal growth and pathfinding, apoptosis and intra- and inter-cellular signalling pathways. It is also proving to be a powerful model for studying host-pathogen interactions.

1.19.5 Use of cultured parasites for drug testing.

Parasitic nematodes cannot yet be raised in continuous culture, though maturation of larvae to egg laying adults has been obtained (Stringfellow, 1986) e.g with *Nippostrongylus brasiliensis*. However other adult nematodes such as *Ascaris lumbricoides* can only be studied for brief periods outside of the host, due to unsuitable conditions for prolonged i*n vitro* culture. It would be of enormous benefit to helminth research if parasitic worms could be raised in the laboratory throughout their lifecycle as this would enable a greater potential to study their biology and observe the effect of drugs. Systems for maintaining adult stages in culture, following isolation from the host, are plagued by a continuous drop in viability, complicating the interpretation of most drug toxicity tests (Geary, 1999), with the exception of neuromuscular "rapid onset," drugs. Variation in culture success is seen with some species e.g, *Trichostrongylus colubriformis* and *Nippostrongylus brasiliensis*, which are more robust in culture than *Haemonchus contortus*.

Recently it has been possible to use *in vitro* cultures of *Schistosoma mansoni, S. japonicum*, *Hymenolepis diminuta* and *Rodentolepis* (*Hymenolepis*) *microstoma* to carry out drug testing (Behnke, 2000). The entire life cycle cannot be maintained *in vitro*, but certain stages of the life cycle (e.g. schistosomulum or cysticercoid) can be maintained for a period of time long enough to test drug activity. Short-term *in vitro* cultures of schistosomes and cestodes are generally found useful in drug screening.

Aims of Project.

Currently the situation of chemotherapy in human disease due to helminths is reported as satisfactory (Harder, 2002). However owing to a lack of interest, there are relatively few new anthelmintics becoming available (Geary, 1999). In the veterinary field, this is a problem owing to an increase in resistance (Prichard, 2002; Conder and Campbell, 1995) and there remains a potential risk of resistance occurring in humans. A new broad-spectrum anthelmintic would have many advantages, not only in easing treatment of helminth infections in the veterinary field but would also help ease the increasing pressure in humans.

As mentioned, the 5-nitrothiazole, nitazoxanide is active against a wide range of protozoan parasites (e.g. *E. histolytica, G. intestinalis, T. vaginalis, H. pylori* and *C. parvum*) and bacteria. NTZ however, has also demonstrated good activity against helminths with good cure rates being demonstrated with *A. lumbricoides, T. saginata* and *F. hepatica.* However, the activity of this drug against helminths is not yet fully understood. This study was therefore designed to investigate the i*n vitro* anthelmintic mechanism of nitazoxanide, its metabolites tizoxanide and tizoxanide glucuronide and the derivatives, denitro-nitazoxanide, denitro-tizoxanide and 2-benzamido-5-nitrothiazole.

To achieve this objective the design aimed at:

1) Developing an *in vitro* assay to examine acute activity of NTZ and related drugs in helminths using *Caenorhabditis elegans* as a model. Then from initial findings to develop hypotheses which could be followed up.
2) Using information derived from the model to investigate the mechanism(s) of the anthelmintic effect of the drugs in the parasitic nematodes *Ascaris suum* and the trichostrongylids *Nematodirus spathiger* and *Haemonchus contortus.*
3) To extend the investigation into other Classes of helminths using *Hymenolepis diminuta and Hymenolepis microstoma* from the Cestoda and *Schistosoma mansoni* and *Schistosoma japonicum* from the Trematoda. A comparison of NTZ mode of effect between the three classes of worms could then be made.

CHAPTER 2 – Materials and Methods.

2.1 Introduction.

To investigate the mechanism of action of nitazoxanide (NTZ) and related compounds against helminths, various nematode, trematode and cestode worms were studied. Drug effect on individual classes was examined and these effects were then compared with effects on the other classes. Animal work for the supply of parasitic nematodes was kindly carried out by Keith Hunt (VLA laboratories, Weybridge), for trematodes by Quentin Bickle and Yaobi Zhang of LSHTM and for cestodes by Jerzy Behnke and Jill Brown of Nottingham University.

2.1.1 *Caenorhabditis elegans* culture.

2.1.1.1 Plate culture.

Bristol strain (N_2) wild type *C. elegans* used in this study was kindly provided by G. Joshua of the LSHTM. Cultures were initially maintained in 9cm flat-bottomed petri-dishes (Sterilin) containing NGM (Nematode Growth Medium) agar (NaCl 3g (BDH AnalaR), agar 17g (Sigma), peptone 2.5g (Sigma), cholesterol (5mg/ml EtOH) 1ml (BDH), distilled H_2O 975ml, supplemented after autoclaving with 1ml sterile $CaCl_2$ 1M, 1ml sterile $MgSO_4$ 1M and 25ml sterile potassium phosphate pH 6.0 1M (BDH AnalaR)) (Wood, 1988). Plates were seeded with 100μl of *Escherichia coli* OP50, a leaky uracil-requiring strain (Brenner, 1974) grown in LB (Luria-Bertani) medium (10g tryptone, 5g yeast extract (Oxoid), 10g NaCl: made up to a litre with distilled water and autoclaved) (Wood, 1988). The plates were then inoculated with a few adult worms using a flame-sterilized platinum wire pick and allowed to develop for a seven days in a 20°C incubator.

2.1.1.2 Liquid axenic nematode culture.

The nematodes were washed from the plates with 7 ml M9 axenising buffer (KH_2PO_4 3g, Na_2HPO_4 6g, NaCl 5g, 1M $MgSO_4$ 1ml (BDH AnalaR): made up to a litre with

distilled water) (Wood, 1988) to which was added a mixture of 1.4 ml 4M NaOH (BDH) and 2.1ml NaOCl (70%) (Aldrich). The nematodes were then washed 3 times with the M9 buffer by centrifugation at 144 g for 30 seconds. The pellet, containing eggs (Wood, 1988) was transferred into polystyrene, filter-cap tissue culture flasks (Nunc) in liquid medium, containing 3% yeast extract, 1.5% peptone, 1.5% glucose and 0.5mg of haemoglobin/ml (Sigma) [a stock solution of 5% (w/v) haemoglobin in 0.1M KOH was autoclaved for 10 min to generate a sterile water-soluble growth-promoting peptide mixture] (Vanfleteren *et al,* 1990). Axenic liquid cultures were stored in a 20°C incubator and allowed to develop for 2-3 weeks.

2.1.2 Isolation of Dauer larvae.

For initial assays with NTZ and related drugs, the dauer larval stage of the *C. elegans* life-cycle was chosen as it was easier for this stage to be standardised. Dauer larvae were isolated from starved liquid cultures by differential density sedimentation. Liquid cultures of the larvae were allowed to grow under gentle agitation for 12-14 days at 20°C. 0.5 ml of the culture was then layered on top of 1ml of 15% (w/v) Ficoll PM 400 (Amersham) in 0.1M NaCl. Dauer larvae settle in the lower solution within 5 minutes, whereas other stages remain at the interface (Golden & Riddle, 1982).

2.1.3 Cryopreservation of *C. elegans.*

To prepare frozen stocks, two plates containing predominately starving L_1 and L_2 larvae (i.e. one day after bacteria are exhausted) were used. Worms were washed off into 1ml M9 buffer and equal volume of freezing solution (5.85g NaCl, 6.8g KH_2PO_4, 300g glycerol, 5.6ml NaOH 1M and H_2O to 1 litre). The suspension in 0.5 ml aliquots, was mixed and transferred to freezing vials and placed in a styrofoam box at –70°C cooling at ~1°C/min. Vials were then transferred to a liquid nitrogen container. To recover *C. elegans* from liquid nitrogen, cryo-preserved vials were allowed to thaw until solution melted. Then the contents were gently tipped onto one side of a bacteria-seeded 9cm plate (Wood, 1988).

2.2 Motility assay of *C. elegans*.

Adult and L_3 larvae of *C. elegans* were assayed for susceptibility to nitazoxanide (NTZ), tizoxanide (TIZ), denitro-nitazoxanide (DNNTZ), denitro-tizoxanide (DNTIZ), tizoxanide glucuronide (TIZg) and 2-benzamido-5-nitrothiazole (BZNT) supplied by Romark. A negative control, metronidazole (anti-protozoal) and positive controls, mebendazole, fenbendazole, niclosamide, FCCP, CCCP (to examine effect of protonophores) ivermectin and levamisole, were assayed in parallel for comparative pharmacological effects. Stocks of the drugs were prepared in DMSO to give 0.1, 1, 10 and 50 mg/ml solutions and stored at -20°C. Worms were exposed to drugs in a concentration range of 0.1μg/ml (0.32μM) to 100μg/ml (320μM) (for NTZ). Concentrations of DMSO equal to that in the drug solutions were tested as solvent controls.

C. elegans is actively motile in liquid medium. Drug assay end point was taken to be reduced motility, or paralysis. Paralysis could be mild or severe and transient (worms recovering after a period of time) or continuous. Paralytic effect could be tonic (total), spastic (jerking) or flaccid (limp).

Twenty four well flat bottom culture plates (Invitrogen) were used for the assay. Drug dilutions for each drug were made in growth medium to concentrations of 100μg/ml, 50μg/ml, 20μg/ml, 10μg/ml, 5μg/ml, 2.5μg/ml, 1μg/ml 0.8μg/ml, 0.5μg/ml and 0.1μg/ml. Each well contained 2ml axenic culture medium and drug or DMSO control and inoculated with 10 worms (either adults or L_3 stage). Worms were staged at L_3 by starvation to produce dauer larvae (see 2.1.2) (Golden and Riddle, 1982) and drug assays were carried out in triplicate. Drug effects were monitored under 20x magnification using an inverted microscope (Reichert-Jung, Microstar). Continual microscopic observation was maintained for 4 hours post worm inoculation. Then, half-hourly checks were made for a further 2 hours. A dose response curve was obtained from which the concentration of drug causing motility inhibition in 50% of the worms in 1 hour (MI_{50}) was calculated. The MI_{50} values were calculated from fitting to a sigmoidal log curve of [drug concentration] versus 'response' using Sigmaplot 2000.

2.2.1 Growth of development assay of *C. elegans*.

The effect of Romark compounds (NTZ and TIZ) on the development of *C. elegans* from egg to adult was also investigated. In preliminary experiments, a 1-20µg/ml solution of each drug in DMSO was added to the *E. coli* OP50 strain under sterile conditions with a final DMSO concentration equal to 0.1%. DMSO (0.1% w/v) was used separately as a negative control. Plates were then streaked with 100µl of *E. coli* and incubated at 20°C for 24 hours. Eggs from axenised culture medium were then added to the plates and effect on hatching and development was examined microscopically every 30 minutes during the day for seven days using an inverted microscope at 20x magnification.

For more accurate results, axenised eggs were also injected into liquid medium containing 1-20µg/ml NTZ, TIZ or DMSO (0.1%) and the effect of the drugs on egg hatching and development again observed every 30 minutes during the day for seven days using an inverted microscope.

2.3 NTZ as an agonist or antagonist of acetylcholine (Ach) receptors.

This assay was carried out to investigate the agonistic/antagonistic effect of NTZ/TIZ on the nicotinic or muscarinic (Ach) receptor of *C. elegans*. Experiments using the specific ganglion blockers, pempidine and mecamylamine (Sigma-Aldrich), which block the nicotinic receptors in nematodes (James & Gilles, 1985) were carried out. Similarly, atropine (Sigma) which antagonises muscarinic action, was also used to test whether NTZ/TIZ were acting as muscarinic agonists or antagonists. Pempidine was dissolved in 1M HCl and then water to give a stock solution of 1M. Mecamylamine was prepared in water to give a stock concentration of 5mM. Atropine was dissolved in DMSO to give a stock solution of 10mg/ml.

Using the same motility assay technique as described in section 2.2, *C. elegans* at the L_3 stage were pre-treated with either 10µM pempidine or 10µM mecamylamine for 20 minutes, before the addition of NTZ/TIZ 10µg/ml (33/38µM). Worms were monitored continuously under an inverted microscope for 2 hours. Reverse experimentation was carried out by first exposing the worms to NTZ/TIZ 10µg/ml (33/38µM) for 20 minutes and then 10µM pempidine or 10µM mecamylamine was added. This experiment was

repeated using 10-30μM atropine. Worm motility was assessed in comparison to DMSO solvent controls.

2.3.1 Nicotinic receptor mutant *C. elegans.*

Mutants of *C. elegans* were obtained from the *Caenorhabditis* Genetics Centre (University of Minnesota), courtesy of Theresa Stiernagle. The mutants used were strain 904 (unc38) (S. Brenner) a nicotinic receptor mutant and levamisole resistant. The second strain used was 1072 (unc29) (J. Lewis) a nicotinic receptor mutant and levamisole resistant (weak kinker), and thirdly strain 211 (lev 1) (S. Brenner), also a nicotinic receptor mutant and levamisole resistant. Also used was strain 407 (unc49) (S. Brenner) a GABA mutant (uncoordinated, shrinker).

Using the same culture techniques and drug assay conditions described in section 2.2, the *C. elegans* wild type and 4 mutants: unc29, unc38 lev 1 and unc 49 were exposed to NTZ/TIZ at final concentrations of 1-20μg/ml. Controls of the drug diluent DMSO and levamisole 1-20μg/ml (Sigma) were tested in parallel.

2.4 NTZ: Effect on acetylcholinesterase.

Acetylcholinesterase (AChase) from *Electrophorus electricus* (Sigma) activity was determined using 1mM acetylthiocholine iodide as a substrate in the presence of Ellman's reagent, 1mM 5.5' dithiobis (2-nitrobenzoic acid) in 100mM sodium phosphate, pH 7.0, at 20°C (Hussein *et al*, 1999, Ellman *et al*, 1961). The reaction was continuously monitored by measuring absorbance at 412nm, using a Perkin-Elmer Lambda 5 spectrophotometer and the hydrolysis of ACh was calculated from the extinction coefficient (E = 14000 $M^{-1}cm^{-1}$) of the coloured product, 2-nitrobenzoic acid. One unit of AChase is defined as 1μM of substrate hydrolysed per min at 20°C. Concentrations of NTZ ranging from 1-20μg/ml were included to test for inhibition of AChase. The positive control drug metrifonate (a known AChase inhibitor) was used at concentrations ranging from 1-20μg/ml. DMSO was used as a negative control.

2.5 Effect of NTZ on the muscle of *Ascaris suum*.

2.5.1 Source and maintenance of worms.

Adult *A. suum* were obtained from the University of Southampton courtesy of Lindy Holden-Dye. Worms were originally obtained from an abattoir and stored at 37°C in artificial perienteric fluid (APF) buffer (NaCl 27g, $MgCl_2$ 16g, $CaCl_2$ 3g, KCl 3g, Tris 5g and sodium acetate 5.49g, made up to 1 litre using distilled water). The APF was then made pH 7.6 with ethanoic acid and 4g of glucose was added. *A. suum* can be maintained for up to 4 days *in vitro* changing the APF twice daily.

2.5.1.1 Effect of NTZ on the motility of adult *A. suum*.

Adult worms were individually placed into glass beakers containing 60ml of APF at 37°C. NTZ was added containing 10µg/ml to 100µg/ml of the drug. The assay was carried out for 48 hours and observations were recorded after 10min, 30min, 1hour, 2 hours, 5 hours, 10 hours, 24 hours and 48 hours. Equal concentrations of the drug diluent DMSO were used as comparative controls.

2.5.2 Worm muscle-strip preparation.

Muscle-strip was prepared as described by Maule *et al*, 2001. A female worm (characterised by turgidity, pinkish colour and well defined with reddish lateral lines) was chosen because they are larger and more amenable to dissection and physiological analysis. The gonopore was located, which is situated about one-third of the worm's length from the head. A transverse incision was made approximately 2mm below the gonopore and another approximately 2cm above. A cylindrical segment of the worm was then able to be removed and placed in a dissecting dish containing APF. The segment was then opened up by cutting longitudinally along the length of one of the lateral lines and pinning down the edges of the segment, outer face downward. This exposed the intestine, visible as a soft brown-green tube-like structure situated in the centre of the body-wall segment. The intestine was then gently pulled away from the segment. A second cut was then made along the length of the remaining lateral line effectively separating the muscle into dorsal and ventral muscle- strip preparations, of which the latter included the gonopore. A needle and thread were then used to make loops with which to attach the muscle- strip to the organ bath holdfast and transducer.

The needle was inserted midway between the cut lateral lines and 3mm from the anterior and posterior end of one of the muscle segments. Using the loops, the segment was transferred to the organ bath containing APF buffer at 37°C and attached to a stationary holdfast (in the organ bath) and a force transducer (Fig 2.1). The recorded voltage on the transducer machine was calibrated by using a 1g weight on the thread.

Figure 2.1: <u>Schematic diagram of muscle tension recording apparatus (organ bath).</u>

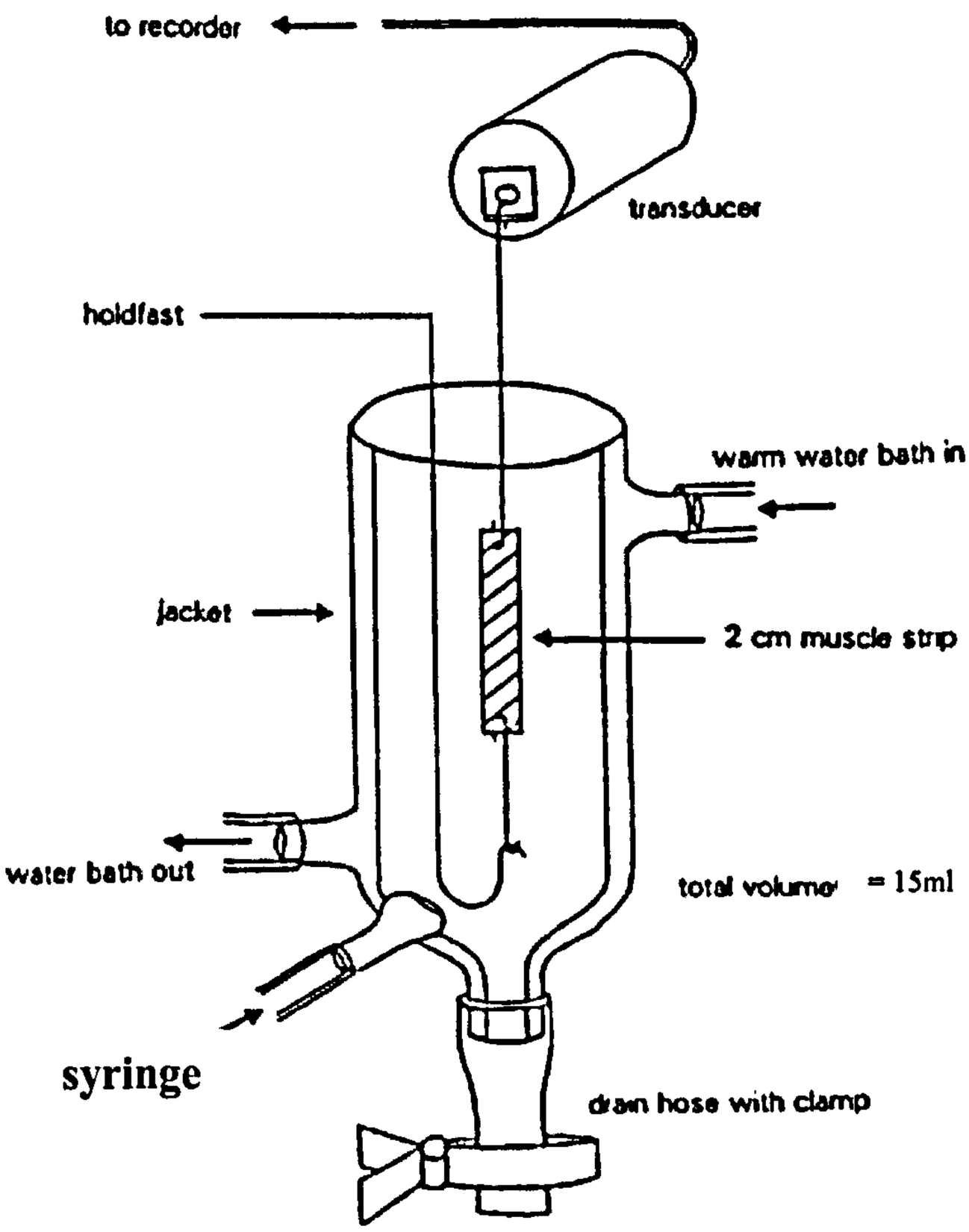


Fig 2.1: Muscle strip is suspended by thread in organ-bath containing 15ml of APF. Drugs at varying concentrations are added via the top of the organ bath.

Diagram from Maule et al, 2001

A syringe containing APF buffer was attached to the side of the apparatus towards the base and when pressed caused agitation inside the organ bath, which assisted rapid distribution and mixing of test compounds. Any movement generated by the muscle was amplified and recorded on a chart recorder. The muscle strip was left for 15min to equilibrate and establish regular spontaneous contractions.

2.5.3 Effect of NTZ/TIZ and acetylcholine on muscle-strip.

2.5.3.1 Effect on nicotinic receptors.

Once the muscle-strip had equilibrated, compounds (ACh, lev, NTZ/ TIZ and DMSO) were tested in turn. Graded concentrations of each compound (10μM to 80μM for Ach and levamisole, 10μM to 600μM for NTZ and 10μM to 100μM for TIZ) were added and the resulting effect was measured on the chart recorder. Equal concentrations of DMSO to those used with the drugs were used as a negative control. Drugs were injected into the organ-bath just below the surface of the APF buffer and 20 minutes contact time allowed before the drug was washed off the muscle-strip by rinsing for 30 seconds with fresh buffer. The bath was then refilled and the experiment repeated with the addition of another dose of the drug.

2.5.3.2 NTZ/TIZ effect on chloride-gated ion channels.

The experimental set up described in 2.5.3.1 was used to examine NTZ/TIZ effect on chloride-gated ion channels. A range of 10μM-600μM NTZ or 10μM-100μM TIZ was used and the drug was added to the bath immediately after addition of 30μM levamisole or 30μM Ach. This experiment was carried out to examine whether rapid relaxation and cessation of contraction occurred. NTZ/TIZ cessation of contraction would suggest the drugs were targeting chloride channels in the worm as has been previously demonstrated for GABA by Maule *et al*, (2001).

2.6 Assay for fumarate reductase using *H. contortus*.

Kuramochi *et al* (1995) suggested that inhibition of nematode fumarate reductase may be a factor in the mechanism of action of nitazoxanide. This was investigated using adult *Haemonchus contortus* as described below.

2.6.1 Isolation of *H. contortus*.

Adult *H. contortus* CVL strain were obtained from Keith Hunt (VLA Weybridge). Worms were collected from a lamb's abomasum post mortem. The abomasum was cut up and worms gently washed off into Earle's Balanced Salt Solution (EBSS) at 30°C (116.4mM NaCl, 5.4mM KCl, 1.8mM $CaCl_2$, 0.4mM $MgSO_4$, 0.9mM NaH_2PO_4, 11.9mM $NaHCO_3$, 50mM MOPS. The medium was then adjusted to pH 7.2 with 5M NaOH). The worms were then washed 5 times in EBSS to clean them from gut flora etc.

2.6.1.1 Effect of NTZ on *H. contortus* motility.

Ten adult worms were assayed in triplicate for NTZ effect on motility in a 24 well culture plate containing 1.5ml of EBSS at 37°C. NTZ was added to 10µg/ml and effect on worm motility and morphology monitored microscopically (magnification 20x) with an inverted microscope for 8 hours. Monitoring was initially every ten minutes for the first two hours, followed by half-hourly checks for the remainder of the assay. An equal concentration of drug solvent (DMSO) was used as a comparative control.

2.6.2 Preparation of *H. contortus* mitochondria.

Adult *H. contortus* mitochondria were prepared essentially as described by Prichard, 1970. The nematodes were homogenised at 4°C in a 1.5ml Dounce homogeniser in mitochondrial medium (1:2, w/v) containing 0.25M sucrose, 0.01M Hepes/KOH pH 7.4, 0.1mM EGTA and 0.1 % bovine serum albumin (fraction V). The homogenate was then centrifuged at 1000 g for 15min at 4°C. The pellet was discarded and the supernatant containing the mitochondria was then centrifuged at 9500 g for 15min at 4°C. The mitochondrial pellet obtained was then resuspended in 5ml of mitochondrial medium and further centrifuged at 16000 g for 15min. The pellet was thereafter resuspended in 5ml 0.1M Hepes/KOH buffer pH 7.4 and stored on ice until required.

2.6.3. Fumarate reductase assay.

Methods described previously by Prichard, (1973) and Bryant & Bennet, (1983) were employed in the fumarate reductase assay. For the assay a lambda-5-spectrophotometer was used (Perkin-Elmer). The spectrophotometer waterbath was maintained at 37°C and a solution of 1mM K-phosphate buffer (pH 7.0) was warmed to the same temperature prior to use. Absorption of the co-factor (NAD-NADH) was measured at 340nm. Determinations of the rate of NADH oxidation were conducted under ambient air in a final volume of 1.0ml containing 1mM buffer, mitochondrial suspension (0.1mg protein) 10μM $MgCl_2$ and 0.3μM NADH.

The reaction was started by the addition of sodium fumarate (final concentration 10μM) and the rate measured. After the reaction had started, varying drugs were added to observe for inhibition. Effects of NTZ and TIZ were examined in a concentration range of 1 to 20μg/ml (3-65μM). Rotenone a known fumarate reductase inhibitor (Bryant & Bennet, 1983) was used as a positive control in the same concentration range. DMSO at comparable concentrations was used as a negative control.

Fumarate reductase activity was calculated by subtracting the rate seen with NADH, drug and Hepes buffer (instead of fumarate) from the rate observed with NADH, drug and fumarate. Statistical analysis was performed using a Student *t*-test where $P<0.05$ was considered significant.

2.7 Larval migration inhibition assay for determination of susceptibility of parasitic L_3 larvae to NTZ and related drugs.

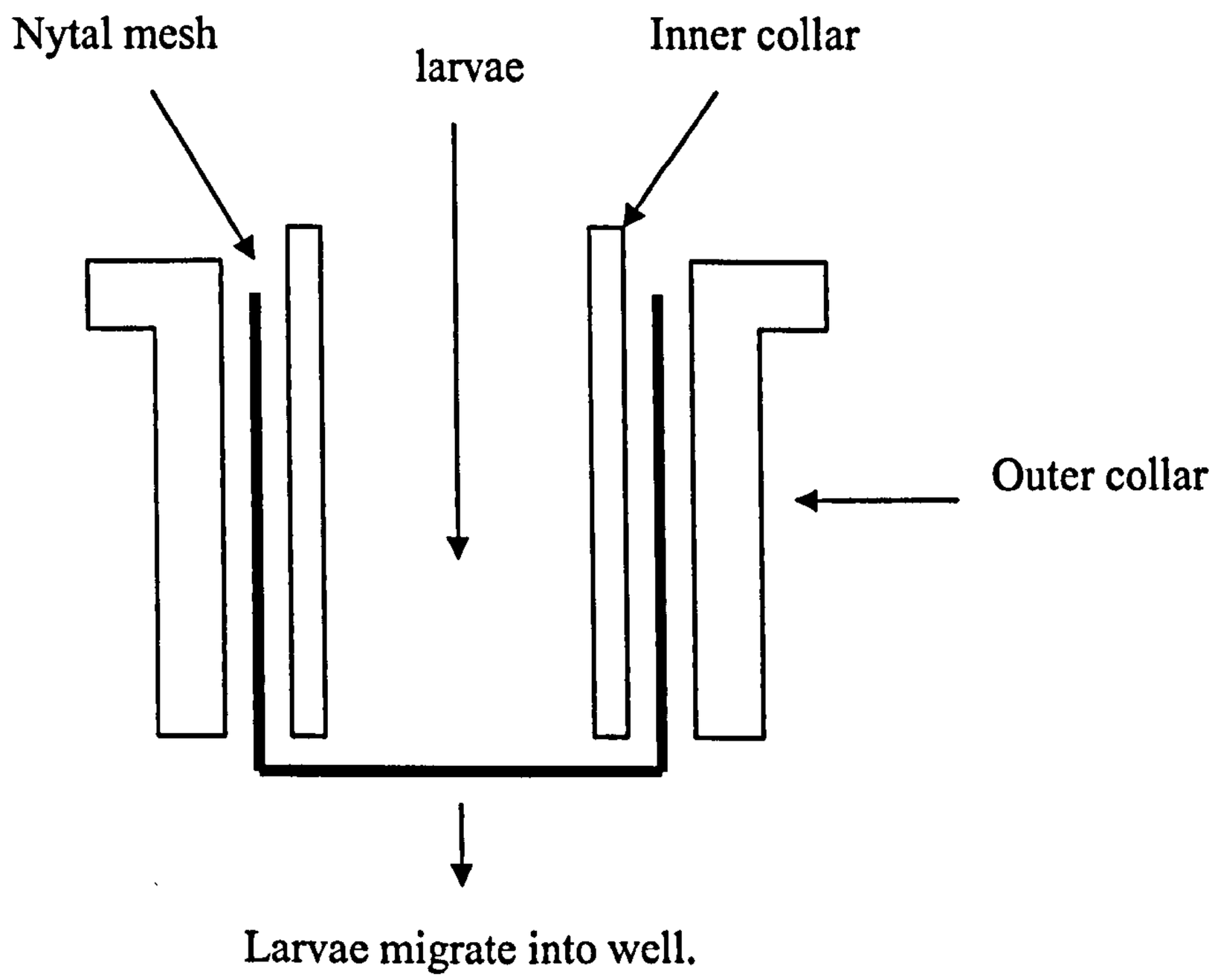
Stage L_3 infective larvae of *Nematodirus spathiger* (CVL strain) and *Haemonchus contortus* (CVL strain) were provided courtesy of Keith Hunt and Ralph Marshall from VLA Weybridge. Lambs were infected by initially keeping in worm-free conditions from birth until the lamb was old enough for infection. An infective dose was given orally in water, 8,000 larvae for *H. contortus* and 20,000 larvae for *N. spathiger.* Infections became patent about 24 days after ingestion. The larvae were obtained by fitting a harness and collection bag to a lamb and collecting faeces. The bag was emptied every 24 hours and the nematode eggs cultured to third stage larvae by incubation in enamel trays for 7 days at 27°C. Coarse faecal material was removed

using a sieve and then larvae were harvested from the faeces by adding warm water (22°C) and using a 'Baermann funnel technique'. This consisted of a high wet-strength filter paper, held over a plastic cylinder and fixed in place with an elastic band. The liquid-larval suspension was poured through the filter paper, temporarily restraining the larvae on the filter. The filter and holder were then immersed in warm tapwater (22°C) allowing larvae to migrate through the filter into the cylinder (Jackson *et al,* 2001). Larvae were then washed and stored in water. L_3 *H. contortus* could be maintained *in vitro* in water at 10°C for 6 months and L_3 *N. spathiger* at 5°C for 1 year in water (1500 larvae/ml) in flat culture flasks.

2.7.1 Design of larval filter apparatus.

A larval filter apparatus was made to a similar design to that used by Jackson *et* al, 2001 (figure 2.2). A filter of Nytal mesh with pore size of 25μm (Lockertex), was cut to 40mm x 40mm and held in place in a polythene cylinder cut from a 2ml pastette, by using an inner collar from a polypropylene plastic pipette tip (Grenier) trimmed to size. Individual filters were then placed into separate wells of a 24 well plate.

Figure 2. 2 Schematic representation of larval migration filter apparatus.



Larvae (still sheathed) were placed into 1.5ml eppendorf tubes, centrifuged for 2 min at 100 g and then washed three times with 0.85% NaCl solution. Larvae were quantitated by taking 100µl samples and counting on a slide using an inverted microscope at 100x magnification. Then larval density was adjusted to retain 100 larvae per 100µl of sample. Motility was measured by the ability of larvae to penetrate the mesh and migrate into the well. Control larva suspensions contained concentrations of DMSO equal to the drug-containing solutions and the migration of larvae from this solution was compared to that from drug treated solutions. Differences in migration were taken to be indicative of a drug effect on larval motility.

2.7.2 Assay of larval motility with selected drugs.

The larval motility assay was modified from those of Jackson *et al*, 2001 and Douch *et al*, 1993. L_3 larvae of *N. spathiger* and *H. contortus* (triplicates) were exposed to each Romark compound (see section 2.2) at concentrations ranging from 0.1 to 30µg/ml (0.3-100µM). Controls included levamisole (positive control), DMSO and 0.85% NaCl (negative controls). Other drugs assayed were niclosamide, mebendazole, CCCP and FCCP. Firstly 200µl of 0.85% NaCl solution (containing approximately 200 larvae) was placed into 1.5ml eppendorf tubes containing 1.3 ml of 0.85% NaCl and the appropriate drug solution. The larvae were then spun down for 15 seconds in a microfuge and the supernatant removed. 1ml of 0.85% NaCl containing an equal drug concentration as before was added and the larvae incubated at 37°C for either 2, 4 or 8 hours. The suspension was then centrifuged and the volume reduced to 200µl. 1.8ml of 0.85% NaCl containing each drug concentration was added to individual wells of a 24 well culture plate (Nunc) and the filter meshes placed into each well, ensuring that the mesh was fully submerged to avoid trapped air bubbles. The larval suspension was mixed thoroughly and the larvae in 200µl of the same concentration of drug as in the well, were added to each filter by gently pipetting down the inner collar. A cover was placed over the plates and larval migration through the filter was allowed to proceed for 2 hours at 37°C. The filters were carefully removed and any remaining larvae on the upper surface, washed into individually labelled petri dishes and stained with a few drops of helminthological iodine/ potassium iodide (Lugol's solution). A few drops of Lugol's solution was also added to each well on the culture plate. The number of larvae in each well and each corresponding petri dish were counted using an inverted

microscope at x40 magnification and percentage migration was calculated for each concentration as indicated below.

$$\% \text{ migration} = \frac{(N_m)}{(N_m + N_r)}$$

Where: N_m = number of larvae migrating through mesh (in well).

N_r = number of larvae retained by mesh (washed off).

A plot of drug concentration against percentage migration was used to determine the IMg_{50} value (concentration at which 50% of larvae fail to migrate) by using Sigmaplot 2000 .

2.7.3 Exsheathment of third stage larvae.

To examine the importance of the larval sheath, larvae were exsheathed and the above assay repeated. For exsheathment, larvae of *H. contortus* or *N. spathiger* were placed into 1.5ml eppendorf tubes and 80µl of 70% sodium hypochlorite solution (Sigma-Aldrich) was added per ml of larval suspension. After 2 min (time needed for all larvae to exsheath) the larvae were centrifuged for 2 min at 100 g and then washed three times with 0.85% NaCl.

2.8 Assay for nitroreductase activity in *C. elegans*.

In this assay, *C. elegans* was used in order to examine whether any nitroreductase activity is present in the worm as suggested by the findings of Bryant & Deluca, 1991. The positive control drug nitrofurazone (NF) used in this assay, is reduced by the nitroreductase of *E. cloacae* and the activity of NTZ/TIZ was measured in comparison to that of NF.

C. elegans plates were grown (as described in section 2.1.1.1) and after seven days, washed 4 times in M9 buffer to remove *E. coli* as described in section 2.9.1. *C. elegans* were made into a final suspension of 2ml axenic medium with glucose, centrifuged at 100 g for 1 minute and the pellet homogenised on ice in a Dounce homogeniser using buffer containing 0.25M sucrose, 0.01M Hepes/KOH pH 7.4, 0.1mM EGTA. Nitrofurazone stock solution 10mM was prepared in DMSO, NADPH and NADH stocks were 100mM in water.

The assay was carried out spectrophotometrically at 37°C at 375nm with nitrofurazone and at 375, 390, 400 and 412nm for NTZ and TIZ. 1 ml of reaction buffer (50mM MES, 100mM Tris, pH adjusted to 7.0 using acetic acid) was added to a cuvette to which was added an enzyme system required for recycling of the nicotinamide nucleotide cofactor. This enzyme system consisted of either: NADPH 0.3mM, glucose 6 phosphate (10µmole) and 1 unit of glucose 6 phosphate dehydrogenase (Sigma), or NADH 0.3mM, 1 unit of alcohol dehydrogenase (Sigma) and 10µl of ethanol. On addition of the enzyme system, the reduced nucleotide absorbance level initially rose and then stabilised. 1, 10 and 20µg/ml nitrofurazone or NTZ or TIZ or the DMSO negative control was then added, causing the absorbance to increase and then level off again. Various volumes of *C. elegans* extract (0.1-0.3mg/ml protein) were then added and any change in absorbance recorded. Protein content of *C. elegans* extract was calculated as described in section 2.9.4.

2.9 ATP inhibition assay.

The method of Ronner *et al* (1999) was modified for the ATP inhibition assay (see 2.9.2). This is a luciferin/luciferase assay system conducted using primarily *C. elegans* and then *S. mansoni*.

2.9.1. Preparation of worms.

C. elegans were grown in 9cm petri-dishes. After inoculation, worms were allowed to develop for two weeks. They were then washed off the plates into 50ml culture tubes with M9 buffer, and then washed to remove bacteria as described by Araújo *et al*, 1999 by adding 20ml of M9 and spinning at 100 g for 1 minute to produce a pellet. The

supernatant was removed and washing repeated four times. After the final wash 5ml of axenic media containing glucose was added to the pellet producing a suspension of bacteria-free *C. elegans*. Individual 50ml culture tubes were used for the assay and drugs at concentrations ranging from 1 to 20µg/ml were added. Drugs tested were NTZ, TIZ, DNNTZ, DNTIZ, FCCP, CCCP and LEV. DMSO (0.2% w/v) and axenic medium were used as a negative controls. Worms were exposed to each drug concentration for 1 hour at room temperature. After this time the tubes were again briefly centrifuged at 100 g to produce a pellet. The drug solution was removed and 2 ml of solubilising reagent: 0.1M NaOH/ 0.5mM EDTA was added. The tubes were then incubated at 60°C for 2 hours to solubilise the worms. Solubilised extracts were then transferred to – 20°C storage and left overnight.

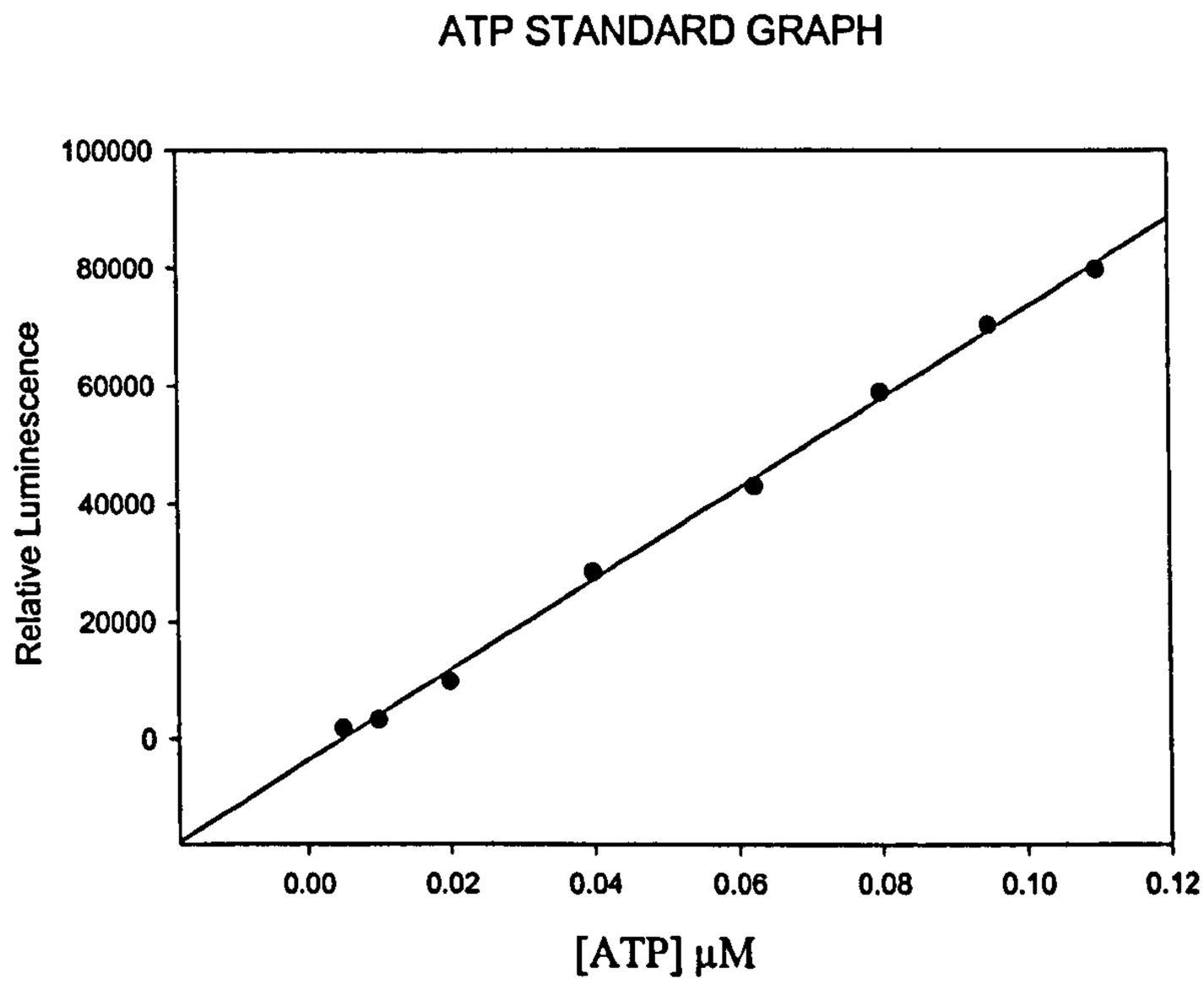
2.9.2 Measurement of ATP inhibition.

A stock solution of ATP assay buffer: 250mM glycylglycine, 2mM EGTA, 2mM $MgCl_2$ and 0.4g BSA per litre made to pH 7.4 with 1M NaOH was prepared and stored in portions at –20° C until required. Upon thawing, a portion of stock solution was made 7.5mM in dithiothreitol. Luciferin (Sigma) and luciferase (Sigma) were added to 15μM and 10μg/ml respectively. The solution was then allowed to stand at room temperature for 2 hours before use. The NaOH/ EDTA worm solutions were thawed and 0.5, 1, 1.5, 2, 3, 3.5, 4 and 5µl were each added to 0.2ml of 0.2M NaOH/ EDTA.

A stock solution of 10µM ATP was made in water and ATP at different concentrations was used as a standard. To make this, 0.1, 0.2, 0.4, 0.8, 1.25, 1.8, 2.2 and 2.8μl of ATP stock solution were added to 0.2ml 0.2M NaOH/ EDTA as per the worm solutions. 30μl of each soluble extract and each ATP concentration were then added to a white flat-bottomed 96 well plate (Wallac) and 106μl of the luciferin/ luciferase solution added to start the reaction. The reaction was measured in a 1450 Microbeta® Scintillation- β counter (Perkin-Elmer, UK) with the machine configured to read luminescence at 10 second intervals.

From the Scintillation- β counter, using Sigmaplot 2000, the ATP standard allowed a curve to be plotted, showing the amount of relative luminescence per each concentration

of ATP. An example of a standard curve is shown below. A separate standard curve was obtained for each experiment.



A curve was also obtained for each solubilised *C. elegans* extract, showing amount of luminescence per sample. Counts of luminescence from the ATP standard were then compared with the *C. elegans* samples in order to find the concentration of ATP in each *C. elegans* extract. A protein control using BSA was then calculated using methods as described in section 2.9.4 to find the amount of ATP/mg of protein in each extract. Inhibition of ATP in the presence of drug compared to the DMSO or medium only negative controls was then examined. Statistical analysis was performed using a Student *t*-test and difference in mean levels of ATP, where $P \leq 0.05$ considered significant.

Controls were also carried out using all concentrations of drug and DMSO with known concentrations of ATP to control for direct effect on ATP.

2.9.3 Preparation of protein reagent.

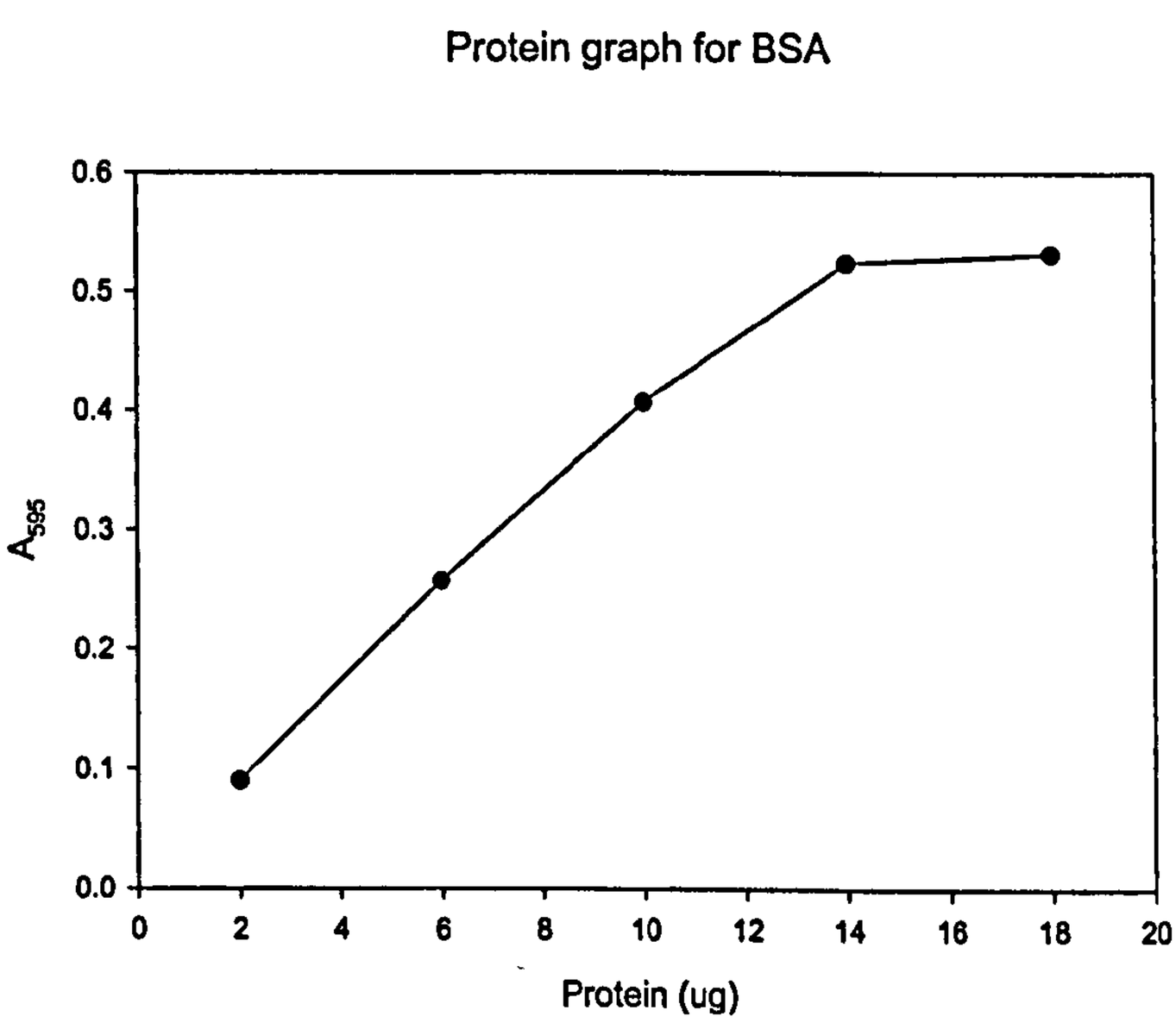
Coomassie Brilliant Blue (G) 250 was used to prepare the protein standard according to Bradford, (1976). 10mg of reagent was dissolved in 5ml 95% ethanol. To this solution 10ml 85% phosphoric acid (w/v) was added and the resulting solution was diluted to a final solution of 100ml and filtered, then stored in a dark, tightly sealed bottle.

2.9.4 Protein assay.

Several dilutions of standard were prepared containing from 1 to 25µg/ml BSA (1mg/ml stock solution). A standard curve was obtained each time the assay was performed.

0.8ml of standards and appropriately diluted samples were placed in clean dry test tubes. 0.8 ml sample buffer or mQ water was placed in "blank" test tube. 0.2ml of Dye Reagent Concentrate was added and vortexed several times. After 1hour, the OD_{595} was measured on a spectrophotometer versus reagent blank. The OD_{595} versus concentration of standards was plotted and the unknown samples read from the standard curve.

Usually six standards of BSA 1mg/ml were added to the 0.8ml mQ each with 2µl/6µl/10µl/14µl/18µl/21µl. For unknowns 2µl (duplicates), 4µl, 8µl 10µl 14µl and 18µl were added. The reading was then recorded. An example of a standard protein curve is shown below. A separate standard was used for each experiment.



2.9.5 Luciferin/luciferase assay for ATP inhibition with *S. mansoni.*

The same assay was carried out as above with adult *S. mansoni.* In this case the worms were incubated for 30 minutes at 37°C instead of room temperature and the assay was carried out in medium 169 (Appendix I). For the assay, 4 worm pairs were used in

1.5ml of medium 169. Praziquantel was used as a control. After freezing/thawing, worm extract was added to a white flat-bottomed 96 well plate and luminescence read as described above. Again a protein assay was performed as described in section 2.9.4 to find concentration of ATP per mg of protein and statistical analyses carried out to examine drug induced differences in ATP levels.

2.10 Trematode susceptibility to NTZ and related drugs.

Since *Fasciola hepatica* was unavailable to study, *Schistosoma mansoni* (and when available, *Schistosoma japonicum*) was used as a model to examine NTZ activity and mode of action in trematodes. Schistosomes were prepared at various stages of the life cycle including miracidia, cercariae, schistosomula and adults.

2.10.1 Culturing of schistosomes.

Schistosomula and adults of a Puerto Rican strain of *Schistosoma mansoni* were obtained from Quentin Bickle of the LSHTM. They were maintained in medium 169 without phenol red (see appendix I) in a 6 well culture plate (Greiner) at 37°C in an incubator with 5% CO_2, 95% air.

2.10.2 Isolation of Adults.

The adult worms were obtained from infected CD1 mice, 20-25g obtained from accredited suppliers and maintained in Category 2 animal facility. For infection, mice were anaesthetized with sodium pentobarbitone and infected percutaneously via a shaved portion of the mouse with 100 *S. mansoni* cercariae freshly shed from *Biomphalaria glabrata* (see section 2.10.5). The cercarial exposure was for 20 mins. On day 49 post-infection, mice were killed with an overdose of sodium pentobarbitone. Thoracic and peritoneal cavities were then opened and 25ml of perfusion fluid (8.9g NaCl, 15g trisodium citrate, 2000 units heparin, 0.2g merthiolate/ litre water) was injected into the right ventricle and the perfusate emerging from an incision in the hepatic portal vein was drained into a plastic 50ml centrifuge tube (Orange Scientific). After allowing parasites to settle, excess solution was removed and erythrocytes in the remaining 1- 1.5ml of fluid were lysed with a few drops of 2.5% saponin (w/v) solution (Doenhoff *et al*, 1978). Worms were then washed 5 times with medium 169 and

transferred to the 6 well plate. Adult *Schistosoma japonicum* (Anhui strain) were provided by Yaobi Zhang, LSHTM using the same procedure (cercariae obtained from *Oncomelania hupensis*) and maintained in the same way.

2.10.3 Collection of *S. mansoni* eggs.

To obtain *S. mansoni* eggs, the liver is removed from the mice after perfusion of the adult worms. Using double strength saline as a wash, the liver was crushed through a sieve with a pestle into a long glass bottle. The crushed liver was then stored at 3-5°C and debris allowed to settle for 20-30 minutes. The saline was replaced, and the settling step repeated. After 20-30min the saline was again removed and cold distilled water added. The remaining liver tissue was placed into a glass Petri dish and eggs identified using light microscopy. Eggs of schistosomes were kept in distilled water at 5°C in the dark until required.

2.10.4 Maintenance of *Biomphalaria glabrata* snails.

B. glabrata snails were provided by Quentin Bickle and Lauren Rattray of LSHTM. Snails were maintained in tanks at 26°C. 5ml of snail salts* was added every 10-14 days. Snails were fed with 3 pellets of food (Special Diets Services) per tank each day. *(100g calcium carbonate, 10g magnesium carbonate, 10g sodium chloride and 2g potassium chloride suspended/dissolved in 3 litres of fresh water).

2.10.5 Hatching of eggs and infection of snails.

In order to hatch the miracidia from the eggs, light (60 watt bulb) was shone directly onto the petri dish. After 10-20min the schistosome eggs within the liver began to hatch and release the miracidia into the water. To infect the snails (*O. hupensis* for *S. japonicum* and *B. glabrata* for *S. mansoni*), approximately 60 uninfected snails were placed into a tank and miracidia added at approximately 20 per snail. After approximately 4 weeks, when the miracidia within the snail would have matured, the snails were kept in the dark for 1 week prior to cercarial shedding. After about 5 weeks after miracidial infection, it was time to shed the snails.

2.10.6 Shedding of cercaria from snails.

The snails of both species were kept in dechlorinated tap water at 24-26°C. Cercariae were shed in a beaker containing a minimum volume of dechlorinated water warmed to 28°C. Shedding was carried out under direct illumination for 30 minutes - 1hour.

2.10.7 Preparation of schistosomula.

Schistosomula were prepared with the assistance of Nuha Mansour (LSHTM) as follows: After shedding the cercariae from the snails as described above, cercariae were mechanically transformed by tail-shedding into schistosomula. This method of transformation of schistosomula from *S. mansoni* cercariae was modified from methods previously described by Ramalho-Pinto *et al*, (1974); Basch (1981); James and Taylor (1976). Cercariae were collected and concentrated in dechlorinated water. 10ml aliquots of the cercarial suspension were then pipetted into 15ml glass conical centrifuge tubes and cooled in an ice bath for 10 minutes to reduce motility of the organisms and facilitate concentration of cercariae (Ramalho-Pinto *et al*, 1974). These were centrifuged at 400 g for 30 to 60 seconds at 4°C and the upper 90% of water was carefully removed leaving behind packed cercariae (Basch 1981). They were then resuspended in 2ml cold E/LAC (Earle's/Lactalbumin) medium (as shown in Appendix I) per tube, and pooled from all the glass tubes into a conical-based plastic 'Universal' tube. E/LAC was supplemented with 300U/ml Penicillin, 300µg/ml Streptomycin and 160µg/ml Gentamycin (Sigma).

Mechanical transformation of cercariae into schistosomula, was done by disruption of cercarial heads from tails by the 'Syringe method' (James & Taylor, 1976). Briefly, cercariae suspended in E/LAC were forced through a 21-guage needle attached to a Luer Lock Syringe a total of 20-22 times. The cercarial heads were left to settle by sedimentation at 37°C under 5% CO_2 and 95% air and then the tail-rich supernatant was carefully removed. Using this technique, the isolated cercarial heads were washed 3 times in 7ml of E/LAC after which they were washed another 2 times with schistosomula culture medium 169 supplemented with antibiotics but lacking foetal calf serum (FCS). Schistosomula were then incubated in medium 169 at 37°C and 5% CO_2.

Transformation and washing were done at room temperature and under sterile conditions.

2.11 Motility and mortality assay of schistosomes exposed to Romark compounds and known schistosomicidals.

Schistosomula (3-10 days old) and paired adult schistosomes (7 weeks) of *S. mansoni* and *S. japonicum* were assayed for susceptibility to the same range of drug concentration and control drugs as used for *C. elegans* (section 2.2). Praziquantel was used as a positive control in the same concentration range. Other known schistosomicidal compounds were also tested for comparison of effect with NTZ/TIZ, e.g. lucanthone, hycanthone, oxamniquine, metrifonate and niridazole as well as compounds with unknown effect (e.g. the uncouplers CCCP, FCCP and proguanil). Other drugs tested included the artemisinin derivatives (artemether, artesunate and dihydroartemisinin), antimycin A, thiabendazole, mebendazole and levamisole. All drugs were in 100% DMSO and stock solutions were made of 10mg/ml, 1mg/ml and 0.1mg/ml. Aqueous drug solutions were kept at -20°C in the dark. The drug diluent DMSO (0.2%) and medium 169 only were used as negative controls.

2.11.1 Assay of adult schistosome activity with NTZ/TIZ and control drugs.

Using sterile technique, adult schistosomes were gently washed 3 times by sedimentation in medium 169 and placed (4 males and 4 females) into wells of a 24 well plate containing 1.5ml medium 169 (in the presence or absence of 10% FCS) and then drug solution added to give required concentration between 0.1µg/ml and 20µg/ml. Each drug was tested in triplicate wells and the plates were maintained at 37°C in 5% CO_2 / 95% air during the assay. The effects of the drugs were recorded for both concentration and effect over time, and examined using an inverted microscope (20x magnification) at regular intervals from 10mins to 120hr. Drug effects were evidenced by motility disturbances (e.g. increased activity or paralysis), morphologic changes (e.g. relaxation, shrinkage, curling) and tegumental effect (disruption, darkening, granulation and blebbing). Death was assumed if no movement (gut or body) was observed during a 2 minute period. Negative control wells contained medium 169 alone or medium 169 with 0.1 and 0.2% DMSO. The pH of the medium was monitored for each assay using a Corning pH meter and was unaffected by any of the drugs added. The assay conditions

used were the same for *S. japonicum* as for *S. mansoni*. LD_{50}'s (in the presence or absence of 10% FCS), showing the concentration of drug that killed 50% of the worms after 120 hrs were calculated using Sigmaplot 2000.

Four week old juveniles were also subjected to NTZ, TIZ, TIZg and PRAZ in medium 169. Conditions were the same as described above and motility and morphologic changes were similar to those in the adult worms.

2.11.2 Assay of schistosomula activity.

Using sterile technique, the schistosomula were gently washed 3 times in medium 169 and approximately 50 larvae were placed into individual wells of a 96 well flat-bottomed culture plate (Nunc) containing 100µl medium 169 (again in the presence or absence of 10% FCS) and exposed to each of the Romark compounds. PRAZ and NIC were used in comparison as positive controls. Drugs were added to the same concentration range (initially in a blinded trial) as for the adults and the effects of the drugs again observed microscopically, for both concentration and effect over time, initially 10min to 1 hour at 37°C in 5% CO_2/ 95% air, then for a further 23 hours at regular intervals. Each assay was carried out in triplicate. Drug effects were again evidenced by motility disturbances, morphological changes and tegumental effect.

2.11.3 Test of NTZ/TIZ drug reversibility.

The same assay, was carried out as above, except that after the adults had been exposed to the NTZ/TIZ for 4 hours (2 hours for schistosomula), worms were removed from the drug, and placed into fresh medium. Incubation was continued with regular monitoring for the remainder of the time (120 hours for adults, 24 hours for schistosomula).

2.11.4 Assay of NTZ and praziquantel on egg hatching and miracidial motility.

100µl of water containing approximately 100 eggs were placed into separate wells of a 96 well flat-bottomed culture plate (Nunc) and pre-exposed to NTZ, TIZ, DNTIZ and PRAZ at concentrations of 1µg/ml and 10µg/ml for 30 minutes before illumination allowed. The assay was then carried out under illumination at room temperature and

constantly monitored using the 20x objective of an inverted microscope (Reichert-Jung, Microstar) for 2 hours. Drug effects on egg hatching and miracidial motility was monitored. DMSO was used as a solvent control at the same concentration as in the drugged wells.

2.11.5 Assay of NTZ and praziquantel on cercarial motility.

Fifty cercariae in dechlorinated water were placed into separate wells of a 96 well culture plate and exposed to NTZ, TIZ, PRAZ , NIC or DNNTZ at concentrations of 1 or 10µg/ml The assay was again carried out at room temperature and effects on cercarial motility constantly monitored under the 20x magnification of an inverted microscope for 2 hours.

2.12 Tests of drug effects at nicotinic and muscarinic receptors in schistosomes.

The effect of Romark compounds and control drugs on the neuromuscular systems of schistosomes were examined.

2.12.1 Test of neuro-receptor antagonists on *S. mansoni* adults.

Adult *S. mansoni* were placed into 96 well culture plates containing 100µl of medium 169 and pre- or post-treated with mecamylamine, pempidine or atropine and 10µg/ml NTZ as described for *C. elegans* in section 2.3.

2.12.2 Test of effect at nicotinic receptor in schistosomes using α-Bungarotoxin.

A stock solution of α-bungarotoxin (125µM) (BTX) (Calbiochem) a specific nicotinic receptor agonist (Haughland, 1998) was prepared in medium 169. Adult *S. mansoni* were then exposed to 10-40µM BTX (as described for NTZ in section 2.11) and the effect of BTX on worm motility was observed over 24 hours. BTX was also used in combination with NTZ or TIZ to check for possible antagonistic effect and hence reduction of drug activity.

2.12.2.1 Visual examination of NTZ/TIZ interaction at nicotinic receptors in *S.mansoni* and *S. japonicum* using rhBTX.

In order to visualise nicotinic receptors in schistosomes (adults and schistosomula), fluorescent tetra-methylrhodamine labelled α-bungarotoxin (rhBTX) (Molecular Probes) was used (200μM stock solution in medium 169). NTZ, DNNTZ and BENZ were added separately before and after addition of rhBTX to view drug interaction at the receptor and hence examine potential inhibition of binding of α-bungarotoxin. The fluorophore was examined in the fluorescent microscope using an excitation wavelength of 553nm and an emission wavelength of 577nm.

2.12.2.2 Examination of possible NTZ/TIZ induced inhibition of rhBTX binding in schistosomula.

Schistosomula of *Schistosoma mansoni* (3 days in culture) were placed into a 96 well microtitre plate (Nunc) in 45μl medium 169 (without FCS) and 5μl rhBTX (20μM final concentration). The plate was incubated for 20 min at 37°C in 5% CO_2/ 95% air. 0.15μl NTZ (30μM final concentration) was then added and incubation was carried out for a further 2 hours. The experiment was also carried out with schistosomula exposed to 30μM NTZ for 20 minutes, followed by 2 hours incubation with 20μM rhBTX. Varying concentrations of NTZ were used, ranging from 1-20μg/ml (3μM to 60μM). Controls of NTZ alone, rhBTX alone and DMSO alone were also tested with the schistosomula.

After incubation, worms were washed in medium 169 as follows:- medium 169 (100μl) was added to the well and the top 100μl of this solution was then removed leaving schistosomula at the bottom of the well and the process repeated 5/6 times to remove the unbound rhBTX. The schistosomula were then fixed with 100μl of 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min. Most of the fixing solution was then removed using a pipette and 200μl of 45% glycerol in 1 x PBS (7.3 g NaCl, 2.36g Na_2HPO_4, 1.31g $NaH_2PO_4.2H_2O$ and H_2O to 1 litre (pH 7.0-7.2)) (Wood, 1988) was added. 5μl of solution containing schistosomula was then transferred onto wells of a immuno-fluorescence multi-well microscope slide (Scientific laboratory

supplies) with cover slip. The slide was examined using a fluorescent microscope (mercury lamp and filter) and confocal fluorescent microscope (Zeiss) (excitation 543nm (green), emission 565nm (red)) with the assistance of Courtney Meier.

2.12.2.3 Examination of possible NTZ/TIZ induced inhibition of rhBTX binding in adult *S. mansoni* and *S. japonicum*.

Effects of drug interaction with rhBTX was also examined using adult *S. japonicum* and *S. mansoni*. Again the protocol was carried out as for the schistosomula with either 20 minute pre- or post drug incubation. The known protonophore CCCP was also tested with adult worms for comparison with NTZ effect. Controls of NTZ alone, rhBTX alone and DMSO alone at the relevant concentrations were also tested with the adult schistosomes.

The BTX and rhBTX experiments were also carried out with *C. elegans* with slight modifications. The stocks of BTX and rhBTX were prepared in axenic media. Drug concentrations were as with the schistosomes but incubation was at 20°C. After incubation, M9 buffer was used for washing away any unbound rhBTX.

2.12.3 Drug effect on muscarinic receptors in schistosomes.

A stock solution of pirenzepine Bodipy Red (fw 805) (BoP) (1.3mM) (excitation 560 nm, emission 569nm) was made in DMSO. The same protocol was used for pirenzepine Bodipy Red as for rhBTX with slight modifications as follows:- Adult *S. mansoni* were placed in a 96 well plate containing 98.7μl medium 169 and 1μl Bodipy (6.5μM final concentration) and incubated for 20 min. NTZ 0.32μl (final concentration 10μg/ml) was then added and the *S. mansoni* were incubated as with the rhBTX for 2hrs at 37°C. The worms were then washed in medium 169, fixed and put on a microscope slide as described earlier. Similarly 10µg/ml NTZ was added to *S. mansoni* 20 minutes prior to addition of 6.5µM BoP, followed by 2 hours incubation. *S. mansoni* with NTZ alone, BoP alone and diluent concentration of DMSO alone were used as controls.

2.12.4 Spectroscopic test for quenching of rhBTX, and pirenzepine bodipy Red fluorescence by NTZ.

NTZ quenching effects on rhBTX and BoP were tested in the absence of worms. Solutions were prepared in a 96 well flat bottom plate and tested for fluorescence as follows: The solutions were 1) Medium 169 and DMSO; 2) Medium 169 and 45μM NTZ; 3) Medium 169, 20μM BTX and DMSO; 4) Medium 169, 20μM Bodipy and DMSO; 5) Medium 169, 20μM of BTX and 45μM NTZ; 6) Medium 169, 20μM of BoP and 45μM NTZ. The relative fluorescence of these solutions was quantitated using the Spectra Max Gemini fluorescent plate reader (Molecular Devices). Excitation was at 553nm, emission 577nm for rhBTX and excitation 560nm, emission 569nm for BoP, with a cut off for both at 565nm.

2.12.5 Qualitative quenching test for NTZ.

The above solutions 1-6 were also examined qualitatively using a Leica fluorescent microscope, (excitation 543nm (green), emission 565nm (red)). A 5μl sample of each of these solutions was transferred to a clean slide and examined under the 10x magnification of a fluorescent microscope and the relative fluorescence qualitatively assessed by eye.

2.13 Assay for nitroreductase in *S. mansoni.*

As described for *C. elegans* in section 2.8 adult *S. mansoni* were tested for possible nitroreductase activity using a method previously adopted by Tracy *et al*, 1983.

2.13.1 Homogenisation of *S. mansoni.*

Adult *S. mansoni* were homogenised using a glass Dounce homogeniser on ice in Tris-HCl buffer containing 0.3M sucrose at pH 7.5. Homogenate was then centrifuged at 4°C for 15min at 2000 g. The supernatant was assayed for nitroreductase activity. Protein levels were measured as described in section 2.9.4.

2.13.2 Nitroreductase assay with *S. mansoni.*

The spectrophotometric assay for schistosomal nitroreductase activity, was carried out under anaerobic conditions in the presence of an oxygen-scavenging enzymatic system in quartz cuvettes at 37°C. The cuvette contained 1ml of 0.15 mM potassium phosphate buffer (pH 7.5), 0.5mg of schistosome protein and either 0.1,0.5,1, 2 or 5 µl of NTZ or niridazole (10mg/ml stock solution) added. DMSO was used as a negative control. 10µl of glucose-6-phosphate dehydrogenase (Sigma), 0.06 units of glucose oxidase (Sigma) and 1100 units catalase (Sigma) were then added to scavenge oxygen upon glucose addition. The contents of the cuvette were then gassed with Argon to remove oxygen and the cuvette covered with a rubber septum lid. 50µl of 0.3M D-glucose solution (Sigma) was then added to the cuvette (via a syringe) and the purging cycle repeated. After maintaining equilibrium for 5 minutes the reaction was initiated by adding 1.5µM NADH and 15µM D-glucose (purged with argon for 2 minutes), through the lid and measuring the decrease in absorbance, (412nm for NTZ, or 400nm for niridazole) continuously for 3 minutes using a Perkin-Elmer Lambda 5 spectrophotometer. A control mixture lacking NADH and glucose-6-phosphate was used as a reference. One unit of nitroreductase activity was to be defined as the amount of enzyme which catalyzed the disappearance of approximately 16 picomol of substrate per minute.

2.14 Assessment of drug effect on *S. mansoni* tegument by Scanning Electron Microscopy.

Freshly isolated adult worm pairs of *S. mansoni* were placed into individual wells of a 24 well culture plate (Nunc) containing 1.5ml of medium 169 and exposed to 10µg/ml of either NTZ, DNNTZ, BZNT or DMSO (0.2% w/v) for 1 hour at 37°C. Thiocarbohydrazide 1% (1.5µl) (Sigma) was also added to the well in order to increase tegumental definition (Malida, 1975).

2.14.1 Preparation of SEM specimens.

After drug incubation, worms were carefully fixed for 2 hours with 3% glutaraldehyde in 0.2M sodium cacodylate buffer pH 7.4. Fixative was then removed by washing 4 times in 0.2M sucrose, 0.075M sodium cacodylate buffer pH 7.4. Worms were then placed in 1% osmium tetroxide for 1 hour at room temperature and then washed again

with 0.2M sucrose, 0.075M sodium cacodylate buffer pH 7.4 at 4°C and then stored in the same buffer until further processing. SEM was carried out by Maria McCrossan (LSHTM) as follows: Worms were washed in several changes of mQ water and then dehydrated through a series of ethanol dilutions - 20 minutes each in 30%, 40%, 60%, 70% and 90% (w/v) dilutions, and finally 90 minutes in 100% ethanol. The specimens were then subjected to critical point drying in an EMScope CPD750 using liquid carbon dioxide. This was followed by sputter coating in an Edwards S150B fitted with a Au/Pd target.

2.14.2. Specimen examination under SEM.

The specimens were mounted on an aluminium stub and then viewed using a Jeol JSM25S Scanning Electron Microscope. Photographs were taken using Kodak Technical PAN roll film TP 120 ISO 25/15 (black and white). The films were subsequently developed in Kodak D19 developing solution and prints of the negatives were enlarged and printed using Agfa multigrade paper and chemistry.

2.15 Examination of inhibition of glucose uptake in *S. mansoni* on exposure to NTZ and related drugs.

2.15.1 Assay of glucose uptake inhibition in *S. mansoni* schistosomula.

Three day old schistosomula of *S. mansoni* were obtained by tail-shedding of cercariae (see section 2.10.7) and transferred to 24 well plates (Nunc) in medium 169 and allowed to develop for 3 days in a 37°C incubator in 5% CO_2/ 95% air. After 3 days 50 schistosomula were placed into individual wells of a 96 well plate containing 100µl of medium 169. Drugs (triplicates) which included NTZ, TIZ, PRAZ, DNNTZ, BZNT and NIC were then added to concentrations of 5, 10 and 20µg/ml. Samples and solvent controls were then incubated for 30 mins at 37°C. After the incubation 2µl of [^{3}H] 2-deoxyglucose (Amersham Biosciences) containing 2µCi was added to each well and the plate incubated at 37°C for a further 20 mins. A protein assay was carried out as described in section 2.9.4 to determine the protein content of 50 schistosomula.

2.15.2 Harvesting of schistosomula.

[^{3}H] labelled schistosomula were harvested using a 96 well harvester (Tomtec, USA). Excess [^{3}H] 2-deoxyglucose was removed by washing the schistosomula gently through the machine at 1 PSI (pounds per square inch) pressure, 3 times with 1x PBS onto a glass fibre filter mat (Perkin- Elmer, UK). The mat was then microwaved to dry at 300 Watts. A Meltilex (Wallac) containing solid scintillant in the form of wax was then placed onto the mat and the mat placed in a 60°C oven for two minutes to allow the Meltilex to melt. The filter mat was placed in a polystyrene sample bag and heat sealed. The tritium count in wells in the sealed bag were then assayed in a Wallac 1450 Microbeta® Scintillation- β counter (Perkin-Elmer, UK) using 2 minute cycles. The data were analysed for drug-related effects on glucose uptake using the Student t-test.

2.15.3 Assay of glucose uptake inhibition in adult paired *S. mansoni*.

Glucose uptake was also assayed in adult paired *S. mansoni* as follows: 2 pairs of adult worms were placed into separate wells of a 96 well plate containing 100μl of medium 169. NTZ, TIZ, PRAZ, DNNTZ and NIC were added to concentrations of 5 and 10μg/ml and incubated as described above. [^{3}H] 2-Deoxyglucose was then added again as described above. After 20 min incubation, the worms were washed 3 times with 1x PBS and after the final wash PBS removed and replaced with 100μl of Wallac Betaplate liquid scintillation fluid (Perkin-Elmer, UK) and [^{3}H] 2-Deoxyglucose uptake determined by a β-Scintillation counter. Data was again analysed using the Student t-test. Protein concentration for each well was calculated after the reading using the protein assay described in section 2.9.4

2.16 *H. diminuta* and *H. microstoma.*

2.16.1 Isolation of adult worms.

To obtain adult worms, 6-8 cysticercoids of *H. diminuta* or *H. microstoma* were inoculated orally by intubation into the host animal using Wistar rats for *H. diminuta* and CD1 mice for *H. microstoma*. Three weeks later adult tapeworms were obtained from the animal's intestine by dissection. Intestines of infected rats/mice were removed and syringed with Earle's balanced salt solution (EBSS) at 37°C, thus displacing any

worms present in the gut. Worms were maintained in EBSS for approximately 48 hours at 37°C.

2.16.2 *Tribolium confusum* (Wild Type) colony.

T. confusum "flour beetles", obtained from Jill Brown (University of Nottingham) were maintained at 22 – 26°C under a fairly dry atmosphere (usually a storage room) in culture medium containing stone ground brown wheat flour, plain white wheat flour (100g:100g) and 5g of brewers' yeast powder. After mixing, about 3cms of flour mix was placed in the base of a glass culture jar (10:10:20 cm, width/breath/height). Circulation of the air within the jar was maintained via small holes in the jar's plastic lid. The beetle culture was placed into the jar, and a cone-shaped piece of filter paper added to allow the beetles to escape from the flour. The beetles lay their eggs in the flour and upon hatching, the larvae grow shedding their skins several times until they pupate and emerge as adult beetles.

2.16.3 Infection of beetles and *in vitro* culture of cysticercoids.

Adult beetles were fed with eggs present in the gravid segments of the adult worm. The segments were excised and ground in a glass dish to make a pulp. The pulp was then fed to beetles that had been left to starve for 5 days in a glass petri-dish layered with filter paper. The pulp was placed on the filter paper allowing the beetles to feed on it. The pulp was left as the only source of food for 2-3 days to ensure it was completely eaten. Cysticercoids could thereafter be obtained from the infected beetles after 21 days. Beetles were maintained by removal of any new larvae. This was done every 2-3 weeks to ensure that the new beetles did not replace the infected ones as they died.

2.16.4 Preparation of cysticercoids.

Healthy looking *T. confusum* beetles previously infected with cysticercoids of *H. diminuta* and *H. microstoma* were selected to ensure presence of living cysticercoids. Two to 3 ml of filter-sterilised insect Ringer's solution (6.5g NaCl, 0.14g KCl, 0.12g $CaCl_2$, 0.1g $NaHCO_3$, 0.01g Na_2HPO_4: Made up to 1 litre with de-ionised water) was placed into a solid watch glass (radius =2cm).

Using fine-forceps a beetle was transferred to the watch-glass. Under a dissecting microscope the beetle's head was removed by gently pulling with the forceps resulting in the release of the haemocoel content into the insect Ringer's solution. Once dissected the cysticercoids (usually between 5-10 per beetle) were readily apparent within the haemocoel fluid. The cysticercoids were then transferred to another watch-glass containing filter-sterilised Hanks saline (8.0g NaCl, 0.4g KCl, 0.14g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.1g $MgCl_2.6H_2O$, 0.06g $Na_2HPO_4.2H_2O$, 0.06g KH_2PO_4, 1.0g glucose, 0.35g $NaHCO_3$ and 100mg phenol red indicator: Made up to 1 litre with de-ionised water) and the process repeated until approximately 100 cysticercoids were collected.

2.16.5 Hatching of cysticercoids.

Cysticercoids were hatched according to a method described by Behnke, (2001). Five solutions A-E were prepared as follows: A, 2g of pepsin (Sigma) in 100ml of 0.85% NaCl; B, 1.0ml conc. HCL in 100 ml of 0.85% NaCl; C, 1g of trypsin (Sigma) in 100ml of Tyrode's saline; D, 1g of sodium tauroglycocholate (Sigma) in 100ml of Tyrode's saline, and E, Tyrode's saline (8.0g NaCl, 0.20g KCl, 0.20g $CaCl_2$, 0.10g $MgCl_2$, 0.05g NaH_2PO_4, 1.0g glucose, 1.0g $NaHCO_3$/litre).

10ml of 1% pepsin in acid 0.85% NaCl was prepared by adding 5ml of solution A to 5ml of solution B in a small Petri dish (acid pepsin solution). 10ml of 0.5% sodium tauroglycocholate was also prepared by mixing 5ml of solution C and 5ml of solution D in a separate Petri dish (Tyrode's tauroglycocholate-trypsin solution). These mixtures were then warmed to 37°C in a waterbath.

The 100 isolated cysticercoids were transferred to a fresh watch glass containing 2ml of Hank's saline. Cysticercoids in groups of 20 were selected and each placed in separate watch-glasses. Excess fluid was removed using a Pasteur pipette until minimal Hank's saline remained containing the cysticercoids. 2ml of the pre-warmed acid pepsin solution was then added, the watch glass was then sealed with parafilm and incubated at 37°C in a waterbath for 5-10 minutes. The acid pepsin solution was then removed and the cysticercoids washed 4 times in Tyrode's (solution E). After the Tyrode's was removed, 2ml of Tyrode's tauroglycocholate- trypsin solution was added. The

watchglass was then recovered in parafilm and placed back into the waterbath for up to 30min until all cysticercoids were hatched.

2.16.6 Test of effects of NTZ and related drugs on newly excysted cysticercoids of *H. diminuta* and *H. microstoma*.

Ten cysticercoids (newly hatched) were placed into separate wells of a 96 well plate containing 100μl of Dulbecco's Modified Eagle's medium (DMEM). Initially, drug concentrations of 1μg/ml to 10μg/ml were added to wells. Drugs included Romark compounds, niclosamide, FCCP, CCCP, PRAZ and DMSO as a solvent control. The plate was kept in a 37°C incubator (5% CO_2/ 95 % air) and monitored at ambient temperature using an inverted microscope (magnification 20x) every 10 minutes for one hour followed by half-hourly checks for a further 3 hours. Effects on cysticercoids were also examined at 12 hours and 24 hours post drug addition. Drug effects were evidenced by changes in motility, sucker movement and morphology, characterised by scolex damage. MI_{50}'s were found for each drug by making 2 fold dilutions from 10µg/ml and calculated using Sigmaplot 2000.

Similarly compounds were added to cysticercoids in medium containing 10% FCS in order to observe effect of serum on drug effect.

2.16.7 Effect of NTZ on motility of adult *H. diminuta*.

Adult *H. diminuta* were individually placed in plastic 9cm Petri dishes (Sterilin) containing 15 ml of EBSS containing 1 or 10μg/ml of either NTZ, TIZ, DNNTZ, BZNT, NIC or 0.2% DMSO. The worms were monitored continually for a period of 4 hours using a bench dissecting microscope (Nikon). Drug effects were evidenced by motility disturbances (e.g. increased activity or paralysis), morphologic changes (e.g. relaxation, shrinkage, curling), tegumental effect (disruption, darkening, granulation and blebbing) and scolex damage. Death was assumed if worms demonstrated no movement within a 2 minute period.

2.17 Effect of NTZ and selected 'uncouplers' on the oxygen uptake of *H. diminuta*.

2.17.1 Isolation of mitochondria.

Adult *H. diminuta* were obtained from Wistar rats by manual removal from the intestine and maintained in Tyrode's balanced salt solution. 5g of worms (wet weight) were transferred to 25ml isolation medium (5ml of medium per g wet weight of worms) at pH 7.4*. The parasites were cut up finely with scissors and then homogenised with a Dounce homogeniser at 0°C. The homogenate was centrifuged at 1000 g for 15min at 4°C. The supernatant was taken and centrifuged at 9500 g for a further 15min. The resulting crude mitochondrial pellet was resuspended in 5ml of isolation medium lacking BSA then centrifuged at 16000 g for 15min. After washing gently in isolation medium minus BSA the final mitochondrial pellet was resuspended in reaction medium containing 0.15M sucrose, 10mM Tris, 20mM KH_2PO_4, 30mM KCl and 5mM $MgCl_2$ pH 7.4.

* Isolation medium [0.25 M sucrose, 25mM Tris, 1 mM EGTA and 1% defatted bovine serum albumin].

2.17.2 Protein assay.

A mitochondrial sample of *H. diminuta* was assayed for protein content using BSA as described in section 2.9.4.

2.17.3 Measurement of oxygen uptake.

Oxygen uptake was measured at 37°C using a Dual Digital Model 20 oxygen electrode (Rank Brothers LTD) in a similar method as described by Yorke & Turton, (1974). A 0.2ml mitochondrial suspension in reaction medium containing 0.24 mg of protein was added to 0.8ml of reaction medium. Succinate and ADP substrates were added from stock solutions of 160mM and 100mM respectively. After stabilization of oxygen levels for 5 minutes, 6.7mM succinate was added followed by 200μM ADP. The respiratory control ratio was calculated as the ratio of oxygen uptake following and prior to, the addition of ADP. The rate of O_2 uptake was calculated in ng-atom/min/mg protein. NTZ or TIZ were added to see whether coupled respiration and ATP synthesis by the

mitochondrial respiratory chain system was inhibited. NTZ and TIZ (1mg/ml stock solutions) were added to the reaction mixture after addition of ADP. The final concentration range of drug added was between 1 and 20μg/ml. NIC, CCCP and FCCP were used as positive controls and levamisole and DMSO (0.2% w/v) were used as negative controls. All experimental drugs and control drugs were added to the same final concentrations.

2.18 *H. diminuta* tegument preparation for SEM.

Adult *H. diminuta* were exposed as in 2.16.7 to 10μg/ml NTZ, DNNTZ, BZNT, or DMSO. Drug exposure was for 30 min and incubation was at 37°C. After 30 minutes drug-incubation the worms were immediately fixed with 3% glutaraldehyde in 0.2M sodium cacodylate buffer pH 7.4 and a 1cm^2 section of proglottid removed approximately 2cm below the neck of the worm. The specimens were then prepared for SEM using the same techniques described in section 2.14.1 Specimens were examined under SEM and photographed as described in section 2.14.2.

2.19 NTZ as a molluscicidal agent.

Dreyfuss *et al*, (1996) and Rondelaud & Dreyfuss, (1996) have previously described the antimolluscal properties of niclosamide and BZNT. In a modification of the assay used, NTZ and TIZ were examined for potential molluscicidal properties.

2.19.1 Comparative assay of NTZ and niclosamide for molluscicidal activity against *B. glabrata.*

In order to examine molluscicidal drug toxicity, NTZ, TIZ and Bayluscide (70% WP niclosamide) were assayed against *B. glabrata* for effects on snail motility, morphology and viability. For each assay, 10 snails (average size of about 1.5cm in diameter) were placed into separate conical flasks containing one litre of water and the appropriate drug concentration (0.5mg/L or 1mg/L). Each concentration was tested in triplicate. Molluscicide stock solutions were made to 0.25mg/L^{-1} in snail water. 25ml of the stock solution was then diluted with 225ml of snail water to give a working drug concentration of 0.025mg/ml. The assay was carried out for 48 hours, with snails

initially exposed to drug and monitored every 30 minutes for 6 hours. Temperature was monitored periodically and maintained at 26°C for this time After this initial exposure, snails were then washed in fresh snail water, transferred to a recovery container and monitored for a further 42 hours. Food was provided for the surviving snails when they were transferred to the recovery container after 6 hours. DMSO (0.1%) was used in comparison as a solvent control.

CHAPTER 3 – NEMATODES.

3.1 Introduction.

In order to investigate the effects of nitazoxanide (NTZ) on helminths, NTZ and its metabolites (tizoxanide, TIZ and tizoxanide glucuronide, TIZg) and derivatives (denitro-nitazoxanide, DNNTZ, denitro-tizoxanide, DNTIZ, 2-benzamido-5-nitrothiazole, BZNT) were tested on *C. elegans,* a free-living nematode which is simple to culture and which has a fully characterised genome (Wood, 1998). The assay, which included microscopic analysis of the worms in liquid medium was used to examine drug effects on worm motility and behaviour. Using initial findings from *C. elegans*, studies were extended to examine effects on other nematodes: Trichostrongylid sheep parasites, *Nematodirus spathiger* and *Haemonchus contortus* and also *Ascaris suum*, a parasite of pigs.

3.2 Effect of NTZ/ TIZ on *C. elegans.*

L_3 (Dauer) stage larvae, and adult worms of *C. elegans* were assayed to examine NTZ/ TIZ effect on worm motility and viability. Varying concentrations of drug were added to wells of a 24 well plate containing 10 worms (triplicates) in 2 ml of axenic medium at ambient temperature. Effects were monitored microscopically at regular intervals.

Initially worms were exposed to 10μg/ml NTZ (33μM) or TIZ (38μM). At this concentration approximately 90% of adults and L_3 larval stages of *C. elegans* exhibited paralysis. Paralysis was characterised by loss of the normal 'S' shaped movement of the worm and an increase in rigidity producing jerky or spastic movement. It was most marked in the posterior part of the worm. The effect developed over 20 min and lasted for up to 2 hours. The effect was comparable to the "transient spastic paralysis," caused by the anthelmintic compound levamisole (Hardman, 1996). Equivalent concentrations of the drug solvent DMSO produced no observable effect on *C. elegans* motility or viability.

The observed paralytic effect of NTZ/TIZ was concentration dependent with regard to both the proportion becoming paralysed and the rapidity of its induction. At 20μg/ml paralysis was observed in 100% of worms and at 0.1μg/ml no motility effect and hence no paralysis was observed. For those worms demonstrating a paralytic effect, no significant variation in speed of onset (approximately 20 mins) was detected between concentrations of 10 and 100μg/ml.

3.2.1 Effect of other Romark compounds on *C. elegans.*

No observable effect on *C. elegans* was produced by TIZg, DNNTZ, DNTIZ or BZNT at concentrations which produced 100% paralysis for NTZ and TIZ. These compounds were also tested at 30μg/ml, but they still failed to visibly affect the worms.

3.2.2 Effect of other anthelmintic drugs on *C. elegans.*

The effects of the known anthelmintics levamisole, ivermectin, mebendazole, fenbendazole, praziquantel and niclosamide were examined for comparison of the effect observed with NTZ and TIZ. The anti-protozoal compound metronidazole, and the protonophores (uncouplers) Carbonylcyanide m-chlorophenylhydrazone (CCCP) and Carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone (FCCP) were also tested .

Levamisole, which causes transient spastic paralysis, was selected as a positive control. At 10μg/ml, levamisole produced complete paralysis in 100% of worms within 5-10 minutes but, unlike with NTZ and TIZ, no worm recovery was observed for the duration of the assay. At 1μg/ml, 100% paralysis still developed, though speed of paralysis was less rapid, occurring in 10-15 minutes. Of the other drugs tested, metronidazole (MTZ) an anti-protozoal reductively activated nitroimidazole drug, showed no effect within 6 hours. The other anthelmintic drugs, mebendazole, fenbendazole, praziquantel and niclosamide also showed no effect during the 6 hour test. Addition of 10μg/ml and 1μg/ml ivermectin caused rapid paralysis within 5 minutes. Unlike the paralysis observed with NTZ, ivermectin-induced paralysis was tonic evidenced by a complete lack of worm movement. Again no worm recovery was observed. Ivermectin however, was poorly soluble in DMSO and precipitated in axenic medium, hence results were difficult to interpret as worms would

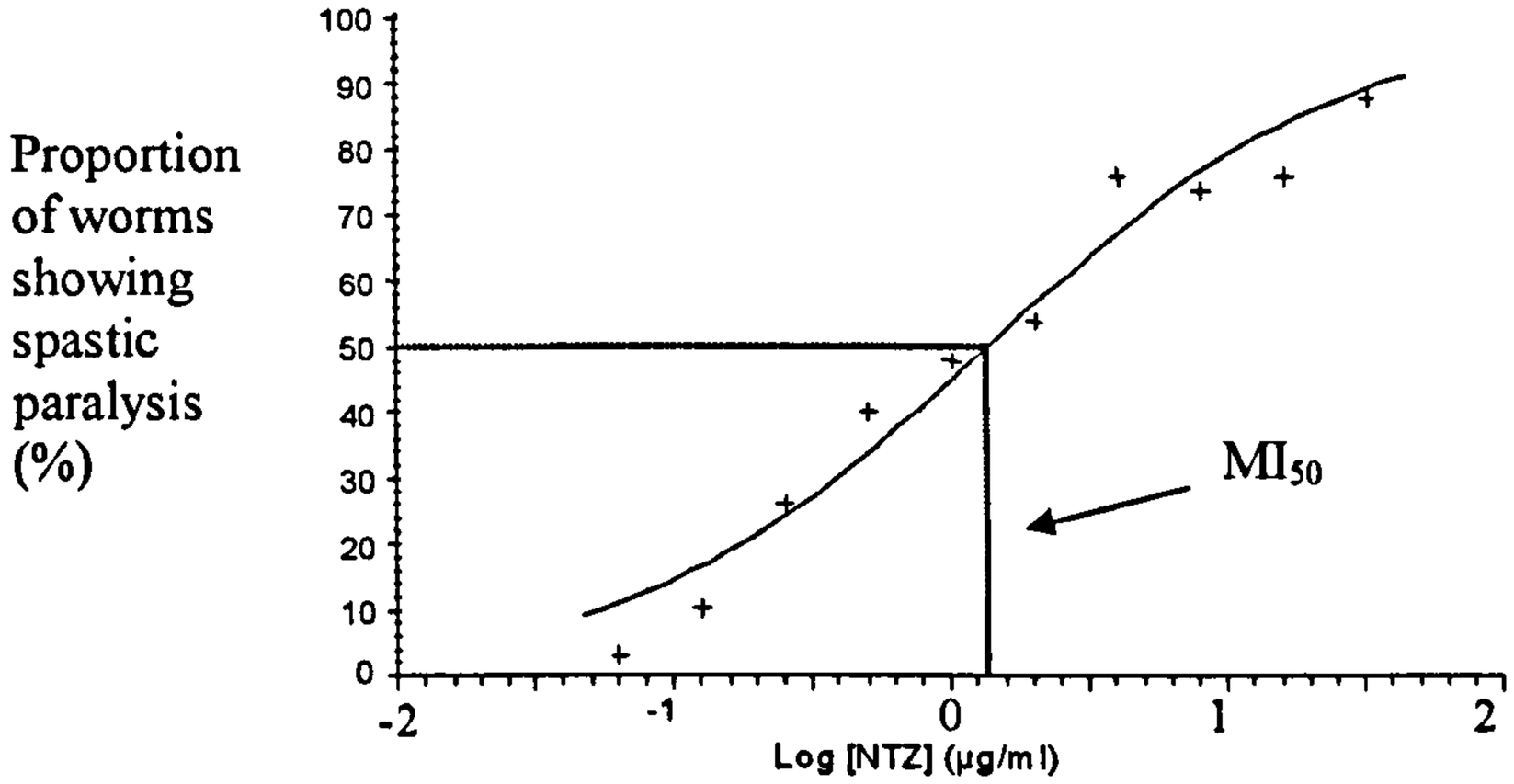
become entangled in the resulting precipitate. Also exact concentrations to induce paralytic effect were unable to be calculated due to this precipitation. Addition of 10µg/ml FCCP or 10µg/ml CCCP caused rapid paralysis (<10 minutes) in 100% of exposed *C. elegans*. The drug-effect was similar to that of ivermectin, in that it resulted in a complete and tonic paralysis of the worm with no movement evident. No recovery was seen in these worms within 6 hours. At lower concentrations (1µg/ml), FCCP had no effect on worm movement with no paralysis evident. Interestingly addition of 1µg/ml and 0.1µg/ml CCCP caused the same rapid and tonic paralysis as observed with the 10µg/ml concentration.

3.2.3 Dose response of NTZ/TIZ in *C .elegans.*

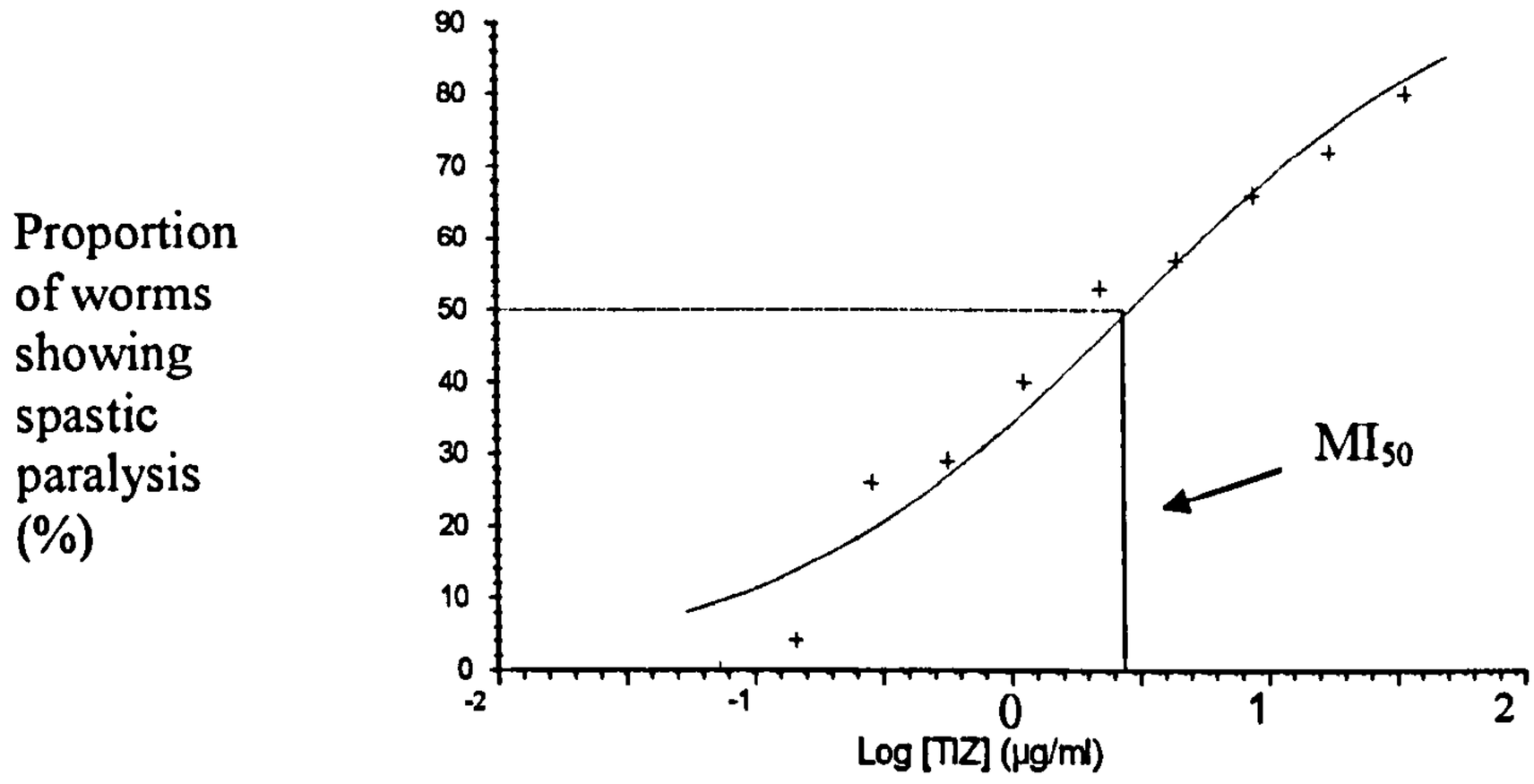
Dose response curves were obtained for NTZ and TIZ for L_3 larvae (figure 3.1) and MI_{50} values (concentration of drug to causing motility inhibition in 50% of worms after 60 minutes) were calculated for these positive Romark compounds using Sigmaplot 2000 (Table 3.1).

Figure 3.1 Determination of MI_{50} of NTZ and TIZ with *C. elegans*.

a).



b).



Paralysis of worms exposed to NTZ (a) or TIZ (b) for 60 minutes in axenic medium. Sigmoid inhibition curves were fitted using Sigmaplot 2000.

Table 3.1: Effect of Romark compounds and control drugs on *C. elegans* motility.

Compound	$MI_{50}^{*} \pm$ S. E. (µg/ml)
Nitazoxanide	1.4 ± 0.2
Tizoxanide	2.9 ± 0.5
Denitro- Nitazoxanide	N.E.
Denitro- Tizoxanide	N.E.
Tizoxanide- glucuronide	N.E.
2 Benzamido- 5- Nitrothiazole	N.E.
Mebendazole	N.E.
Levamisole	> 0.1 < 1.0
Niclosamide	N.E.
Ivermectin	> 0.1 < 1
FCCP	> 1 < 10
CCCP	< 0.1
Metronidazole	N.E
Fenbendazole	N.E

MI_{50} = Concentration of drug which results in the motility inhibition in 50% of the worm population in 60 minutes.

N. E. = No Effect: Inhibition of motility not detected up to 30µg/ml.

* for concentration calculations from µg/ml to µM see Appendix IV.

Table 3.1: The sensitivity of *C. elegans* motility to NTZ and TIZ was measured as a percentage of L_3 larvae affected at 60 min in a range of drug concentrations.

3.2.4 Assessment of effects of NTZ/TIZ on egg hatching and larval development.

3.2.4.1 Culture on solid medium.

Petri dishes containing NGM agar were streaked with 100µl *E. coli* liquid culture which contained from 1 to 20µg/ml NTZ or TIZ. The next day eggs of *C. elegans* were placed onto the bacterial lawn and hatching of eggs observed by regular monitoring every 30 minutes using an inverted microscope at 20x magnification. At no concentration of either drug was egg hatching affected and worms developed normally compared to the solvent controls.

3.2.4.2 Axenic cultures.

In case the lack of effect upon hatching was due to metabolism of drugs by *E. coli* prior to addition of eggs, hatching was also tested in liquid axenic medium. NTZ or TIZ (1-20µg/ml) were added to axenic media containing eggs, and cultures were monitored every 30 minutes using an inverted microscope at 20x magnification. At no concentration did the presence of NTZ or TIZ prevent egg hatching and larval development was normal compared to the solvent controls.

3.3 Test of acetylcholinesterase inhibition by NTZ.

The initial observations of paralysis of *C. elegans* by NTZ suggested a neurological response in the nematodes. This could be due to build up of acetylcholine as a result of inhibition of acetylcholinesterase as described for an anthelmintic mechanism of pyrantel (Hardman & Limbird, 1996). Paralysis could also be due to a direct effect on specific neuro-receptors, which is considered to be the anthelmintic mechanism of levamisole (Martin *et al*, 1997) and ivermectin (Vanden Bossche *et al*, 1985).

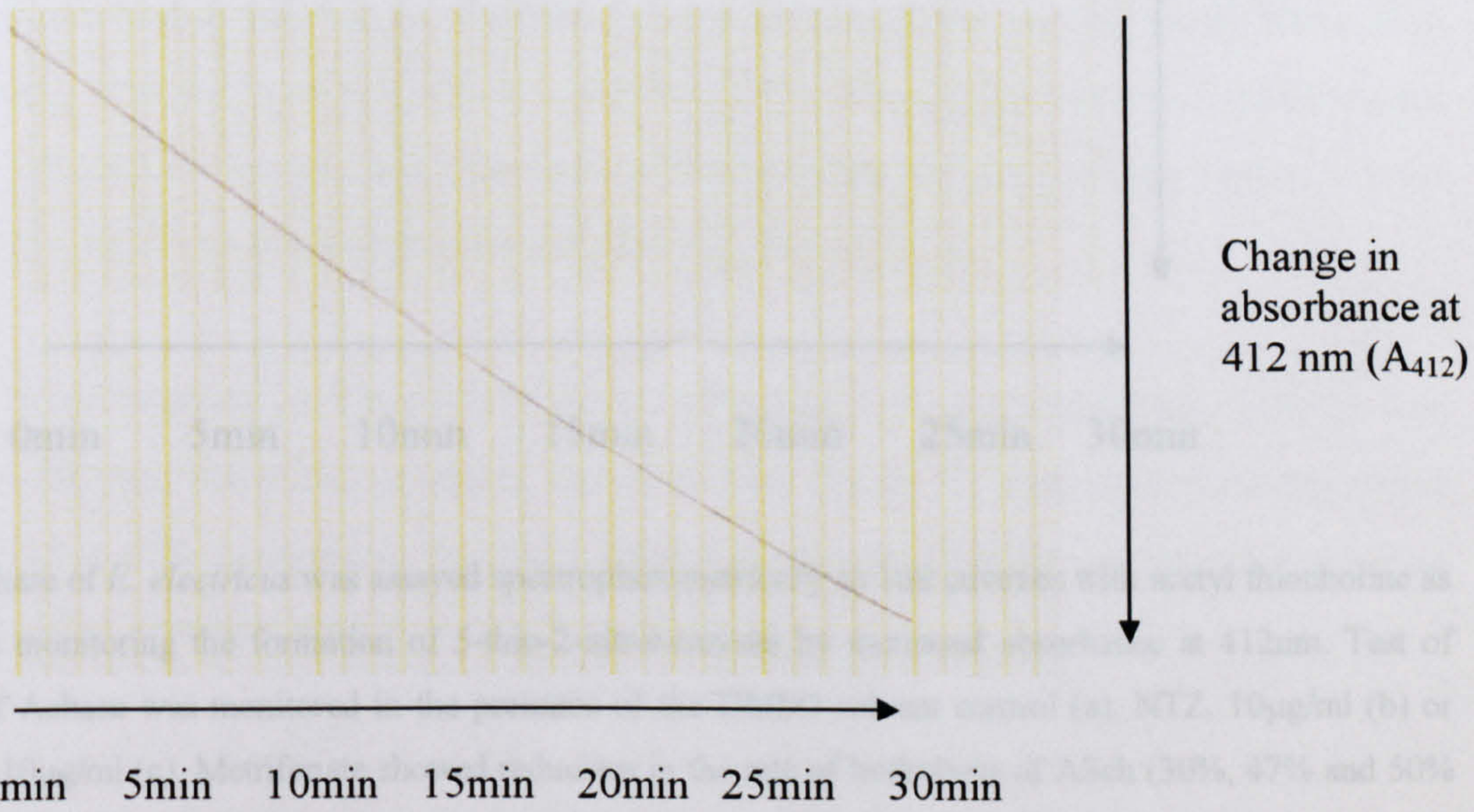
Possible inhibition of acetylcholinesterase (Achase) by NTZ was therefore tested using the type VI-S acetylcholinesterase from *Electrophorus electricus*, which is commercially available and sensitive to inhibition by metrifonate. The enzyme hydrolyzes other alkylacetates, including acetylthiocholine (ASch), which can be observed

spectrophotometrically, in the presence of Ellman's reagent (Hussein, 1999), due to the formation of the yellow 5-thio-2-nitrobenzoate at 412nm.

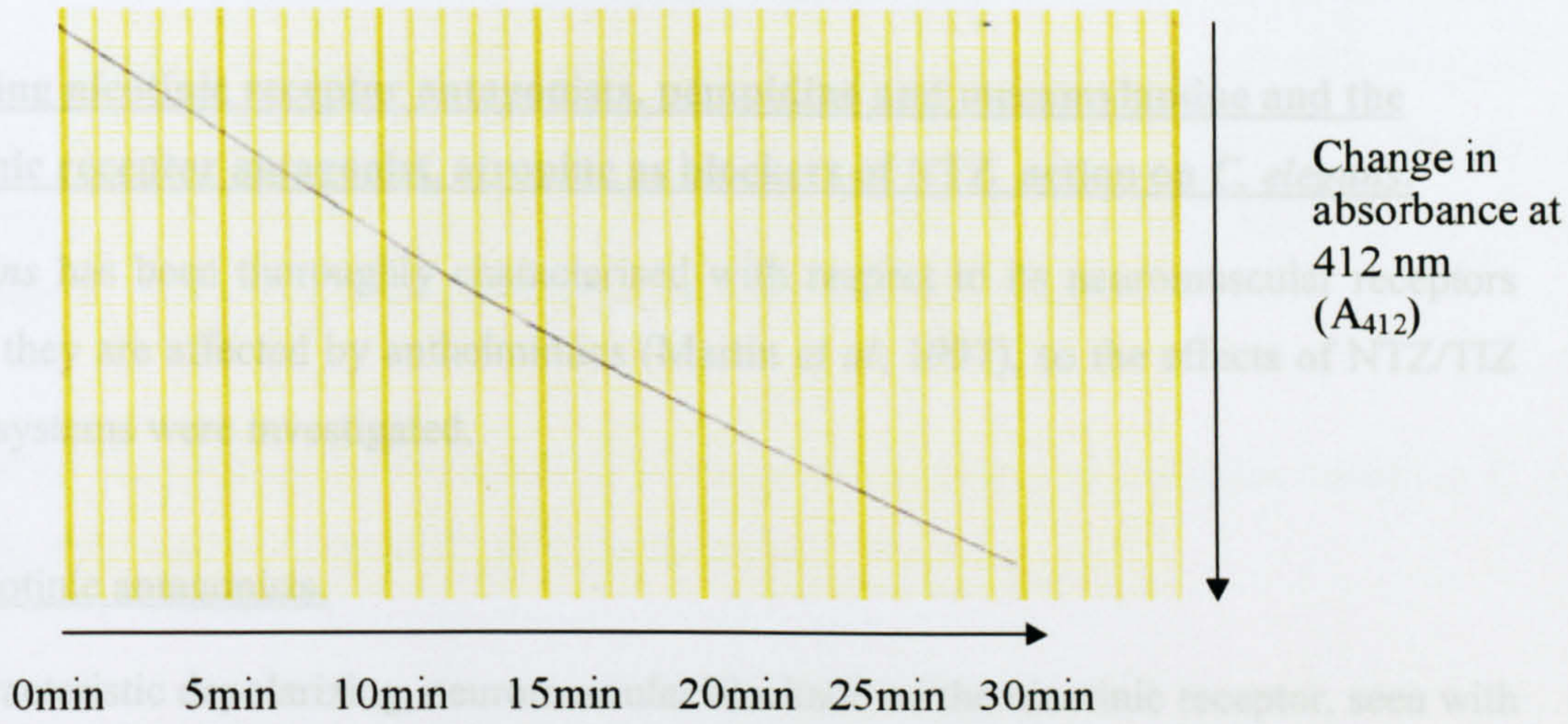
Addition of from 0.1–20μg/ml (0.32μM to 64μM) NTZ in DMSO to *E. electricus* acetylcholinesterase, showed no significant inhibition of rate of ASch hydrolysis over a 30 minute period (see figure 3.2). TIZ similarly showed no effect on the rate. The positive control drug metrifonate (MET) at 10μg/ml clearly inhibited the rate of hydrolysis after a delay of about 15 minutes. This inhibition was dependent on concentration. DMSO only caused no effect in rate of hydrolysis.

Fig 3.2 <u>Test of inhibition of Achase by NTZ.</u>

a) DMSO



b) NTZ 10µg/ml



c) MET 10µg/ml

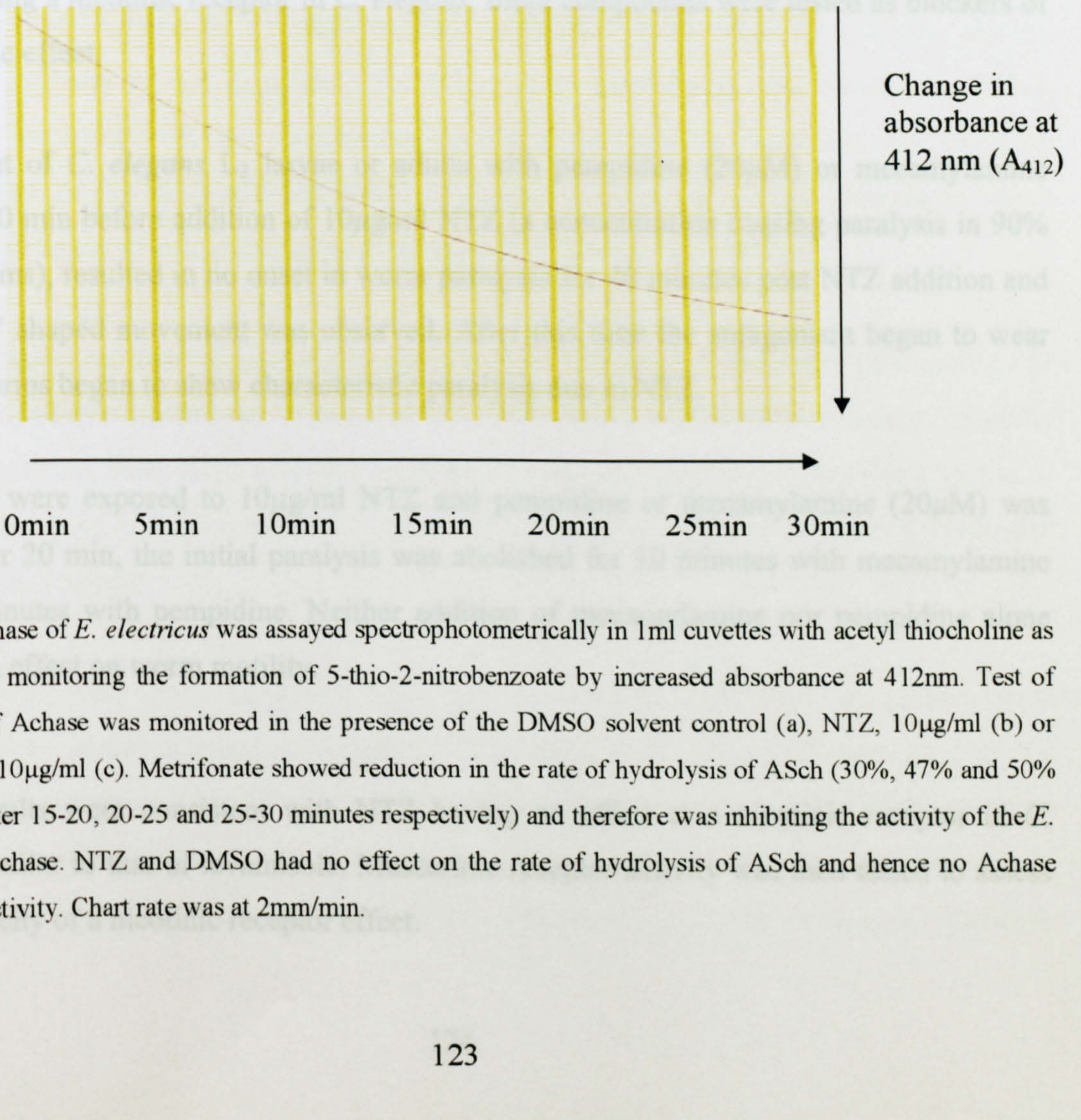


Fig 3.2: Achase of *E. electricus* was assayed spectrophotometrically in 1ml cuvettes with acetyl thiocholine as substrate by monitoring the formation of 5-thio-2-nitrobenzoate by increased absorbance at 412nm. Test of inhibition of Achase was monitored in the presence of the DMSO solvent control (a), NTZ, 10µg/ml (b) or metrifonate 10µg/ml (c). Metrifonate showed reduction in the rate of hydrolysis of ASch (30%, 47% and 50% reduction after 15-20, 20-25 and 25-30 minutes respectively) and therefore was inhibiting the activity of the *E. electricus* Achase. NTZ and DMSO had no effect on the rate of hydrolysis of ASch and hence no Achase inhibitory activity. Chart rate was at 2mm/min.

It was then necessary to test if NTZ caused paralysis in *C. elegans* by direct action on a neuromuscular receptor.

3.4 Testing nicotinic receptor antagonists, pempidine and mecamylamine and the muscarinic receptor antagonist, atropine as blockers of NTZ action on *C. elegans*.

C. elegans has been thoroughly characterised with respect to its neuromuscular receptors and how they are affected by anthelmintics (Martin *et al*, 1997), so the effects of NTZ/TIZ on these systems were investigated.

3.4.1 Nicotinic antagonists.

The characteristic depolarizing, neuromuscular blockade on the nicotinic receptor, seen with levamisole (Martin *et al*, 1997) is blocked by the specific nicotinic, ganglionic- blocking agents pempidine or mecamylamine (James & Gilles, 1985). Therefore, to test whether NTZ was affecting a nicotinic receptor in *C. elegans*, these compounds were tested as blockers of its paralytic effect.

Treatment of *C. elegans* L_3 larvae or adults with pempidine (20μM) or mecamylamine (20μM), 20 min before addition of 10μg/ml NTZ (a concentration causing paralysis in 90% of the worms), resulted in no onset in worm paralysis for 40 minutes post NTZ addition and normal 'S' shaped movement was observed. After this time the antagonism began to wear off and worms began to show characteristic paralysis due to NTZ.

If worms were exposed to 10µg/ml NTZ and pempidine or mecamylamine (20μM) was added after 20 min, the initial paralysis was abolished for 10 minutes with mecamylamine and 20 minutes with pempidine. Neither addition of mecamylamine nor pempidine alone showed an effect on worm motility.

These results were consistent with NTZ having an effect at a nicotinic receptor in *C. elegans* similar to that of levamisole. Muscarinic receptor activity was then tested to assess the specificity of a nicotinic receptor effect.

3.4.2 Muscarinic antagonists.

Addition of the muscarinic receptor antagonist atropine (20μM) to *C. elegans* caused no paralysis or effect on movement of the worm. Pre-treatment and post-treatment with atropine 20 minutes before or 20 minutes after addition of 10μg/ml NTZ failed to prevent or reverse characteristic NTZ-dependent paralysis. This suggests that the paralysis observed in *C. elegans* may be specifically associated with nicotinic receptor activity.

The effect of nitazoxanide on the nicotinic receptor was therefore investigated further in *C. elegans* by examination of specific receptor mutants.

3.5 Analysis of motility inhibition by NTZ in *C. elegans* using specific neuromuscular receptor mutants.

Uncoordinated mutants of *C. elegans* have previously been used to examine macro-molecule function in the nematode nervous system (Richmond and Jorgensen, 1999). Although locomotion in the mutants is impaired, the muscles still contract allowing determination of the mechanism of action of various compounds such as levamisole which specifically targets nicotinic receptors as an agonist (Martin *et al*, 1997).

Neuromuscular mutants of *C. elegans* were obtained to test potential nicotinic activity of NTZ. Assays were carried out to observe NTZ effect in the levamisole-resistant, nicotinic receptor mutants, 904, 1072 and lev1 and these were compared to NTZ effects in the γ-aminobutyric acid (GABA) receptor mutant 407. The wild type and levamisole-treatment were used as controls.

Mutants 407, 904, lev1, 1072 and wild type *C. elegans*, maintained in axenic liquid medium, were each exposed to 10μg/ml NTZ (table 3.2). The wild type showed the characteristic paralysis after 20 minutes. The GABA receptor mutant, 407, which was more rigid than the wild type and moved very awkwardly in axenic media, showed evidence of slight paralysis at the same time as paralysis was observed in the wild type. Nicotinic

mutants 904, lev 1 and 1072, which all have reduced motility compared to the wild type and more jerky movement, showed no evidence of altered motility in the presence of 10μg/ml NTZ. Levamisole (10μg/ml) showed paralysis in the wild type and increased paralysis in mutant 407, which was more clear than that seen with NTZ. In mutants 904, lev 1 and 1072, levamisole showed no effect. Blinded studies were attempted with the mutants, however each mutant had a characteristic type of motility in axenic media and were therefore easy to distinguish between prior to drug addition.

Table 3.2 Effect of NTZ on *C. elegans* nicotinic receptor mutants.

C. elegans strain	Motion	Effect of LEV (10μg/ml)	Effect of NTZ (10μg/ml)
Wild type	'S-shaped'	Spastic paralysis	Spastic paralysis in posterior end
GABA receptor mutant 407	Jerky movement stiff posterior	Paralysis & some curling	Increased jerkiness some paralysis
Nicotinic receptor mutant 904	Slightly spastic kink in posterior	No effect	No effect
Nicotinic receptor mutant 1072	Spastic in movement	No effect	No effect
Nicotinic receptor mutant lev 1	Poor motility stiff posterior	No effect	No effect

Table 3.2 10μg/ml NTZ or LEV was added to mutants of *C. elegans* in 24 well plates (triplicate) containing 2 ml of axenic media at room temperature and effects on motility were observed microscopically for 4 hours. With mutants 1072, 904 and lev 1, spastic paralysis was not observed upon addition of NTZ or LEV during the allotted time. Mutant 407 however, on addition of levamisole became rapidly paralysed and slight curling of the worm was evident. Addition of NTZ caused increased worm jerkiness, followed by sluggishness leading to slight paralysis. Effects however were difficult to determine due to the uncoordinated movement of the mutants.

3.6 Examination of the effect of NTZ at nicotinic receptors in *C. elegans* using tetramethyl-rhodamine-labelled α-bungarotoxin (rhBTX).

Interaction of NTZ/TIZ at nicotinic receptors was further examined in *C. elegans* using rhBTX, a known agonist for nicotinic receptors (Haughland, 1998). In contrast to the results obtained with trematodes (see Chapter 4.), 20 minutes pre-incubation of *C. elegans* with 20μM rhBTX followed by 2 hours incubation with control solvent (0.1% DMSO), examination by fluorescence microscopy showed no binding of rhBTX to the nicotinic receptors or to the surface of the worm. However, the pharyngeal area of the worm showed a large amount of fluorescence, suggesting ingestion of the rhBTX, rather than trans-cuticular entry (not shown). Treatment with NTZ/TIZ 10μg/ml for 20 minutes pre- and post incubation with 20μM rhBTX again showed no binding to *C. elegans* nicotinic receptors or surface marking. Fluorescence was still observed in the pharyngeal part of the worm.

The study of the effects of NTZ on helminths was then extended to the parasitic nematodes *Ascaris suum, Nematodirus spathiger and Haemonchus contortus.*

3.7 Effect of Romark drugs on *Ascaris suum.*

In order to further investigate the effect of NTZ/TIZ on the receptors of nematodes the physiological effects of classical transmitter substances on the motor activity of nematode somatic musculature were examined and compared to those of NTZ/TIZ using *A. suum* (a roundworm similar to *A. lumbricoides*).

Initial experiments on adult *Ascaris suum* motility using NTZ were carried out in glass beakers and effects compared to controls containing equal concentrations of the drug diluent, DMSO in APF. Addition of a final concentration of 40μg/ml NTZ, caused worm behaviour to alter slightly after 15-20 minutes, characterised by the worms coiling themselves into tighter positions. After 50-60 minutes the worms started to become rigid and movement was visibly different to that of the DMSO and APF alone controls. This lack of movement was evident for approximately 2 hours and then worms began to recover slightly. After 24 hours, worms were again sluggish and this sluggishness increased during the remainder of the

assay. Negative controls of worms in DMSO and APF only, were fairly active for the first 24 hours of the assay. After this time all worms showed decreased motility, and sluggishness and no difference could be observed between drug-exposed and control worms after 48 hours. No significant effect was observed with concentrations of drug below 40μg/ml. TIZ (maximum concentration tested 30μg/ml owing to lack of solubility) showed no effect on motility with the adult worms.

3.7.1 Effect of NTZ on *Ascaris suum* muscle-strip preparations.

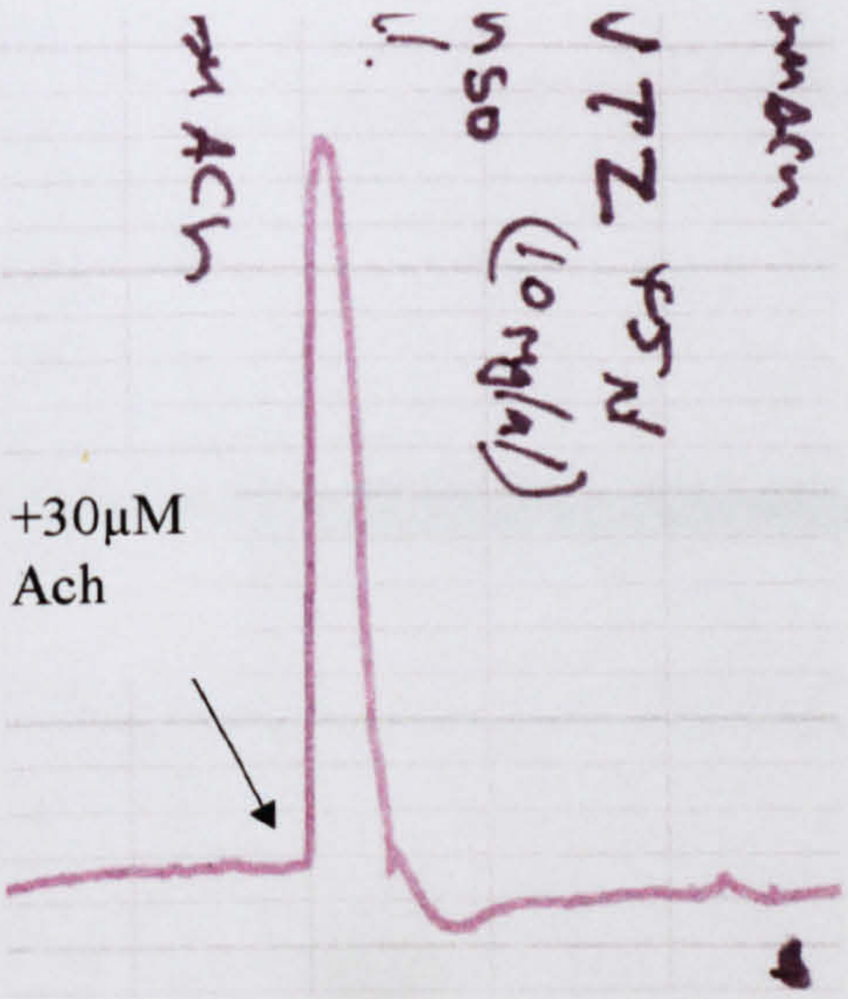
Effects of NTZ were tested on a preparation of dissected *Ascaris* muscle in an organ bath attached to a tension sensor. Muscle contractibility was tested by addition of 30μM acetylcholine (Ach), which caused the muscle-strip to contract resulting in a rapid increase in tension, followed by a rapid decrease due to desensitisation of the tissue (figure 3.3 a). LEV (30μM) also caused a rapid increase in muscle tension, which diminished more slowly (figure 3.3 b). Addition of 150μM NTZ caused a similar contraction of the muscle but it was smaller and diminished more slowly than that observed with Ach or levamisole. Higher concentrations of NTZ (up to a final concentration of 600μM in the bath) increased both the amount of tension in the muscle-strip and the desensitisation period of the drug (figure 3.3 c and d; figure 3.4). The relatively long desensitisation period seen with NTZ suggests a longer persistence of NTZ at the receptor. Concentrations of NTZ below 100μM or the solvent alone (figure 3.3 e) showed no contraction of the *Ascaris* muscle. TIZ up to 100μM, showed no effect on *A. suum* muscle strip. This was the maximum concentration that could be tested due to lack of solubility of this compound.

In order to test whether NTZ/TIZ were interacting with GABA receptors, they were added immediately after addition of Ach or LEV. Under these conditions GABA (30μM) causes rapid relaxation of the muscle strip and cessation of contractions via chloride channel opening (Maule *et al*, 2003). However, neither NTZ (10-600μM) or TIZ (10-100μM) altered the Ach or LEV dependent contractions.

Figure 3.3 Effect of NTZ on *Ascaris suum* muscle tension.

Fig 3.3: *A. suum* muscle strips were tensioned at 1g, and the effect of addition of a) 30μM Ach, b) 30μM levamisole, c) 200μM NTZ d) 400μM NTZ and e) 0.2% DMSO (w/v) final concentration were measured. One horizontal division on the chart is 5 minutes and 8 vertical divisions represent 1g tension. Clear contraction can be seen on addition of 30μM Ach or 30μM levamisole. Addition of 200μM NTZ causes a slight contraction, which increases with increased drug concentration. Equivalent concentrations of drug solvent have no effect on Ascaris muscle.

a)



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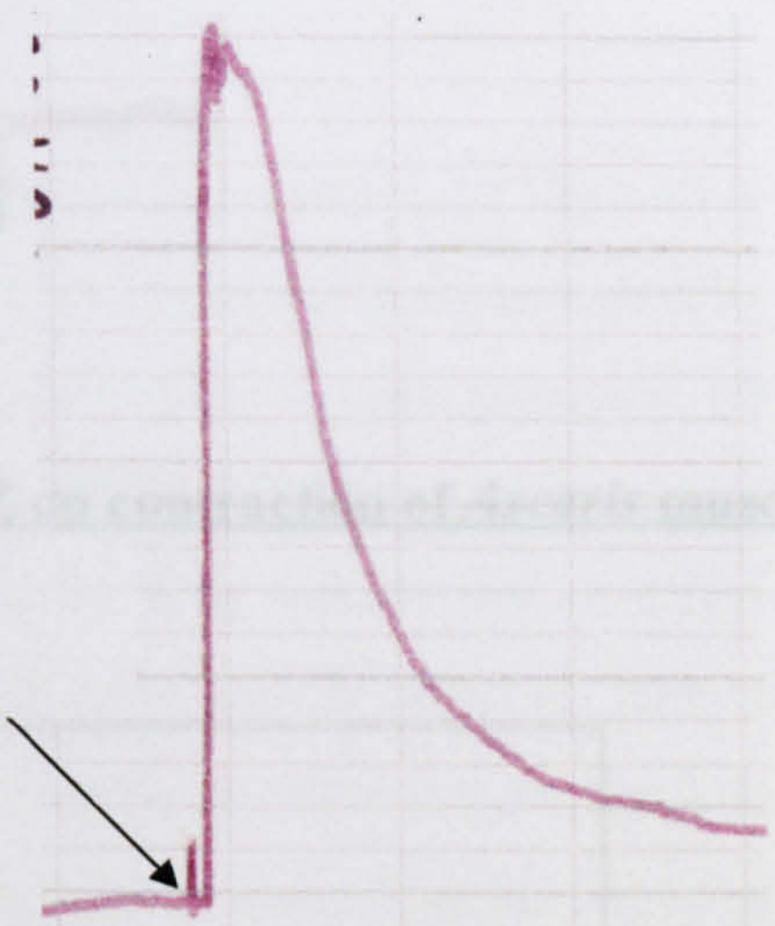

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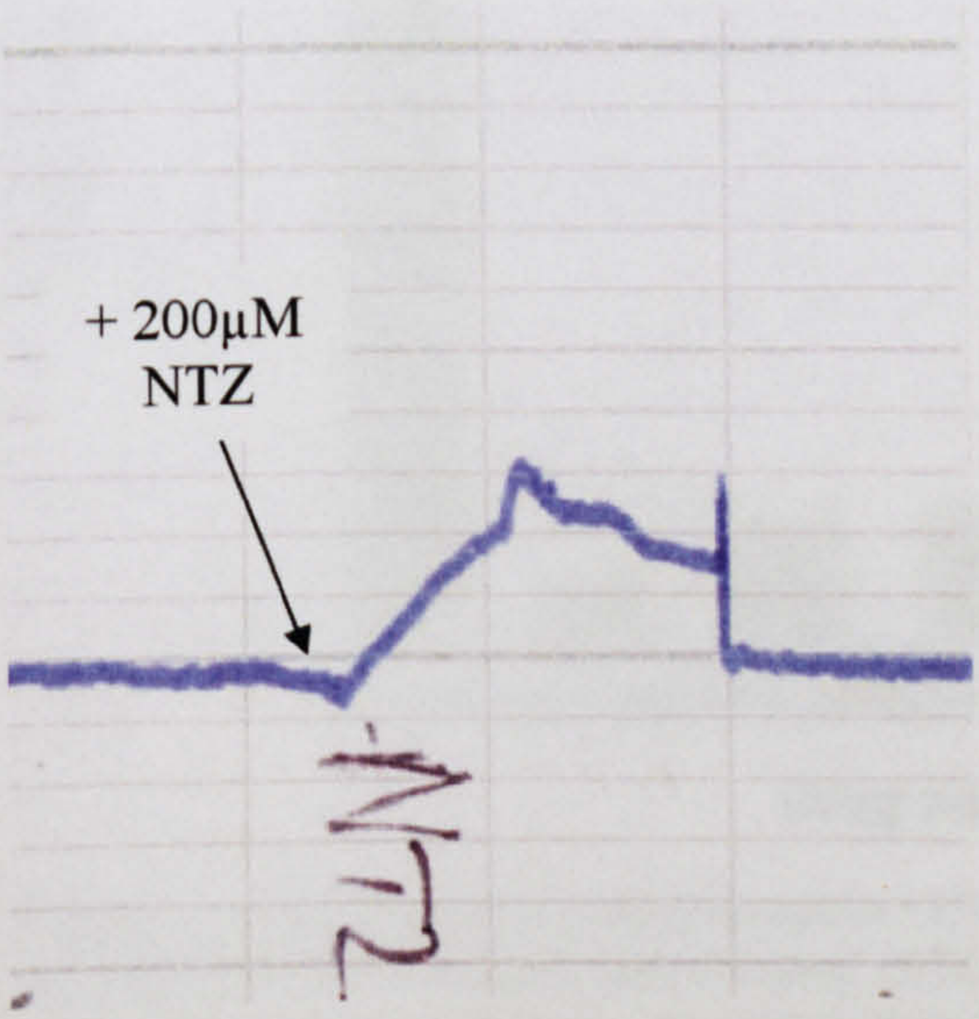

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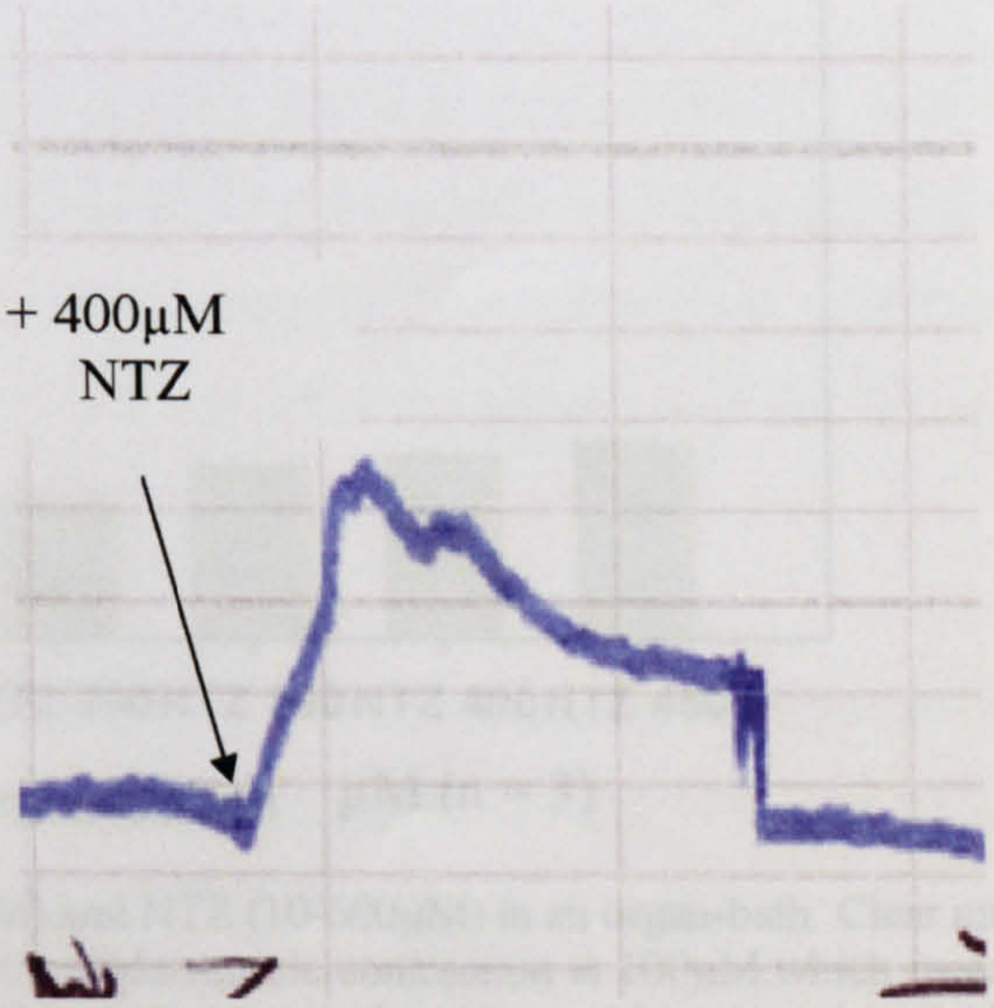


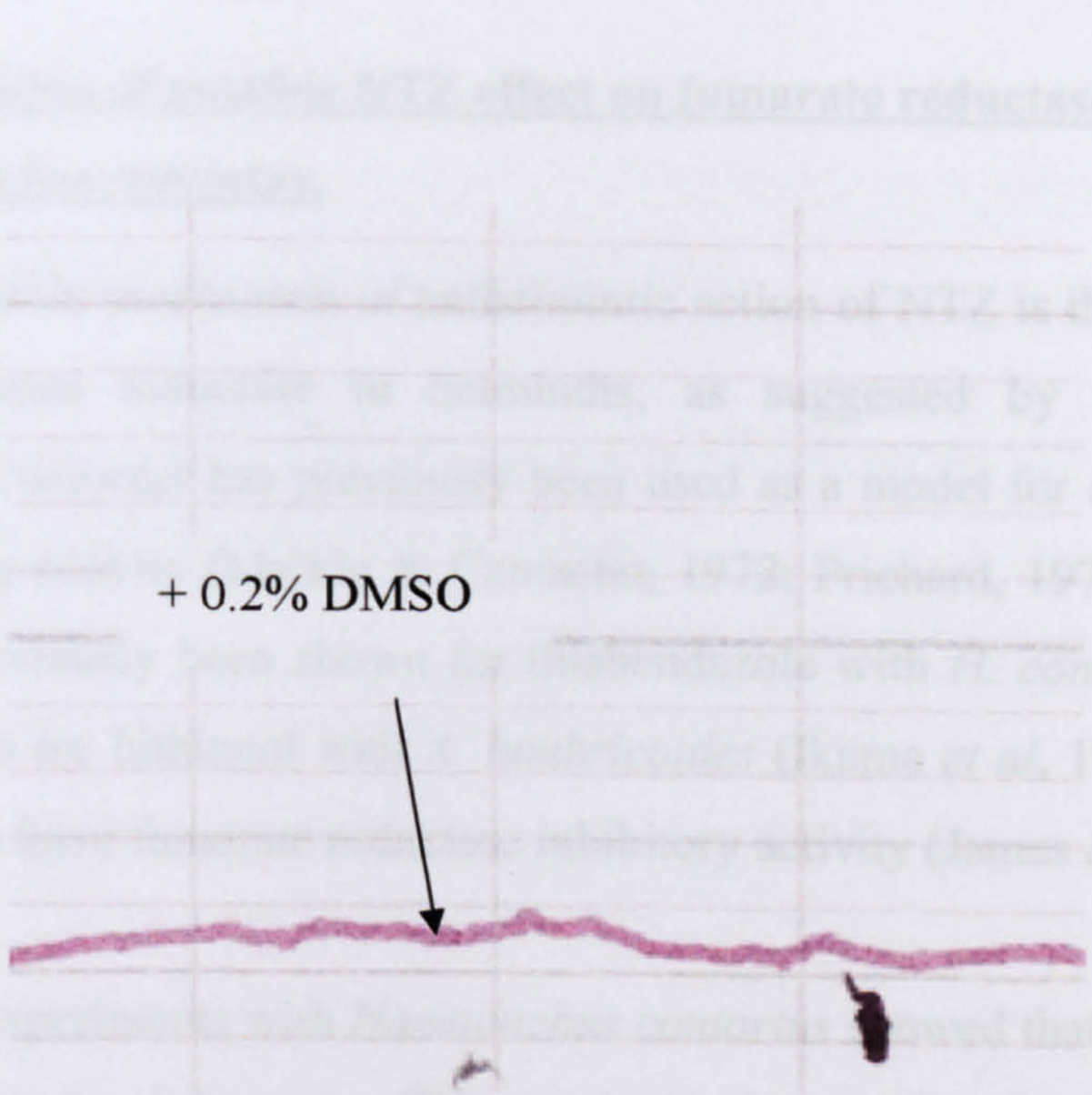


Figure 3.4 Concentration dependence of effect of NTZ on contraction of *Ascaris* muscle.

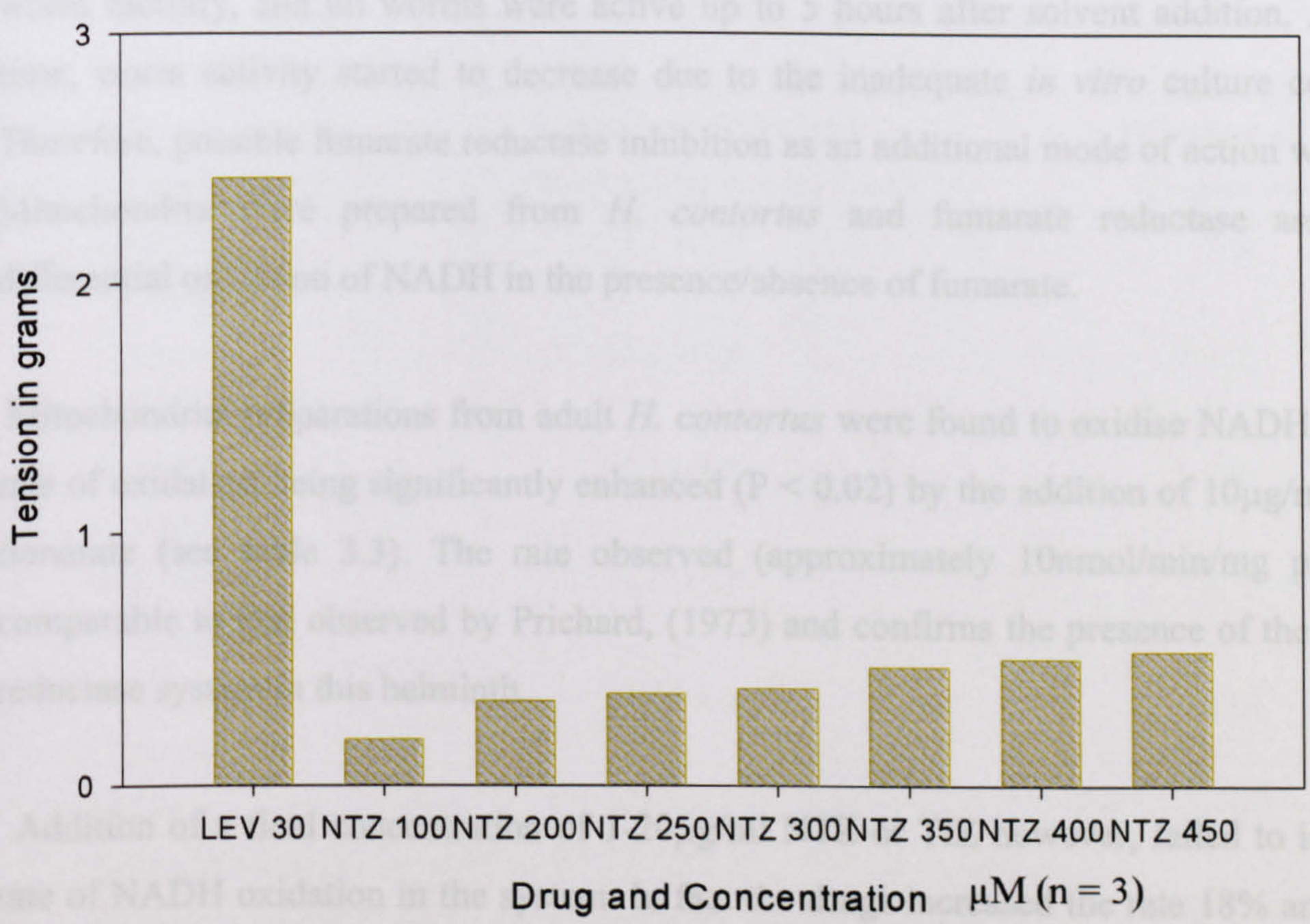


Fig 3.4: *Ascaris* muscle strip was exposed to LEV (30μM) and NTZ (10-600μM) in an organ-bath. Clear muscle contraction with 30μM LEV was observed. NTZ caused a slight muscle contraction at 100μM which increased with increasing concentration. Concentrations of NTZ below 100μM caused no observable contraction. Addition of DMSO only at concentrations equal to those used with the drugs did not cause contraction of the *Ascaris* muscle.

3.8 Examination of possible NTZ effect on fumarate reductase of *Haemonchus contortus.*

Another possible mechanism of anthelmintic action of NTZ is the possibility that the drug inhibits fumarate reductase in helminths, as suggested by Kuramochi *et al*, 1995. *Haemonchus contortus* has previously been used as a model for demonstration of fumarate reductase drug activity (Malkin & Camacho, 1972; Prichard, 1973) and this mechanism of action has previously been shown for thiabendazole with *H. contortus* (Bryant & Bennett, 1983) and also for bithionol with *A. lumbricoides* (Ikuma *et al*, 1993). Levamisole has also been shown to have fumarate reductase inhibitory activity (James & Gilles, 1985).

Preliminary experiments with *Haemonchus contortus* showed that addition of 10µg/ml NTZ caused paralysis in adult worms. This paralysis, characterised by initial jerkiness, developed slowly and was followed by a decrease in worm movement leading to tonic paralysis. 100% of worms were affected within 3 hours. No recovery of worm motility was observed up to 7 hours post drug addition. Equal concentrations of DMSO, the drug solvent, had no effect on worm motility, and all worms were active up to 5 hours after solvent addition. After this time, worm activity started to decrease due to the inadequate *in vitro* culture conditions. Therefore, possible fumarate reductase inhibition as an additional mode of action was tested. Mitochondria were prepared from *H. contortus* and fumarate reductase assayed by differential oxidation of NADH in the presence/absence of fumarate.

Mitochondrial preparations from adult *H. contortus* were found to oxidise NADH, with the rate of oxidation being significantly enhanced ($P < 0.02$) by the addition of 10µg/ml sodium fumarate (see table 3.3). The rate observed (approximately 10nmol/min/mg protein) is comparable to that observed by Prichard, (1973) and confirms the presence of the fumarate reductase system in this helminth.

Addition of a final concentration of 1-20µg/ml NTZ or TIZ however, failed to inhibit the rate of NADH oxidation in the system. In fact the drugs increased the rate 18% and 23% at 10µg/ml respectively. Higher concentrations of NTZ/TIZ also showed a slightly increased rate (see table 3.3 & fig 3.5). Rotenone (10µg/ml), a known fumarate reductase inhibitor

(Bryant *et al*, 1983) significantly decreased the rate of NADH oxidation. Addition of 20µg/ml rotenone, further decreased NADH oxidation rate. DMSO (0.2% w/v) only had a small affect on rate with a 3 % increase observed.

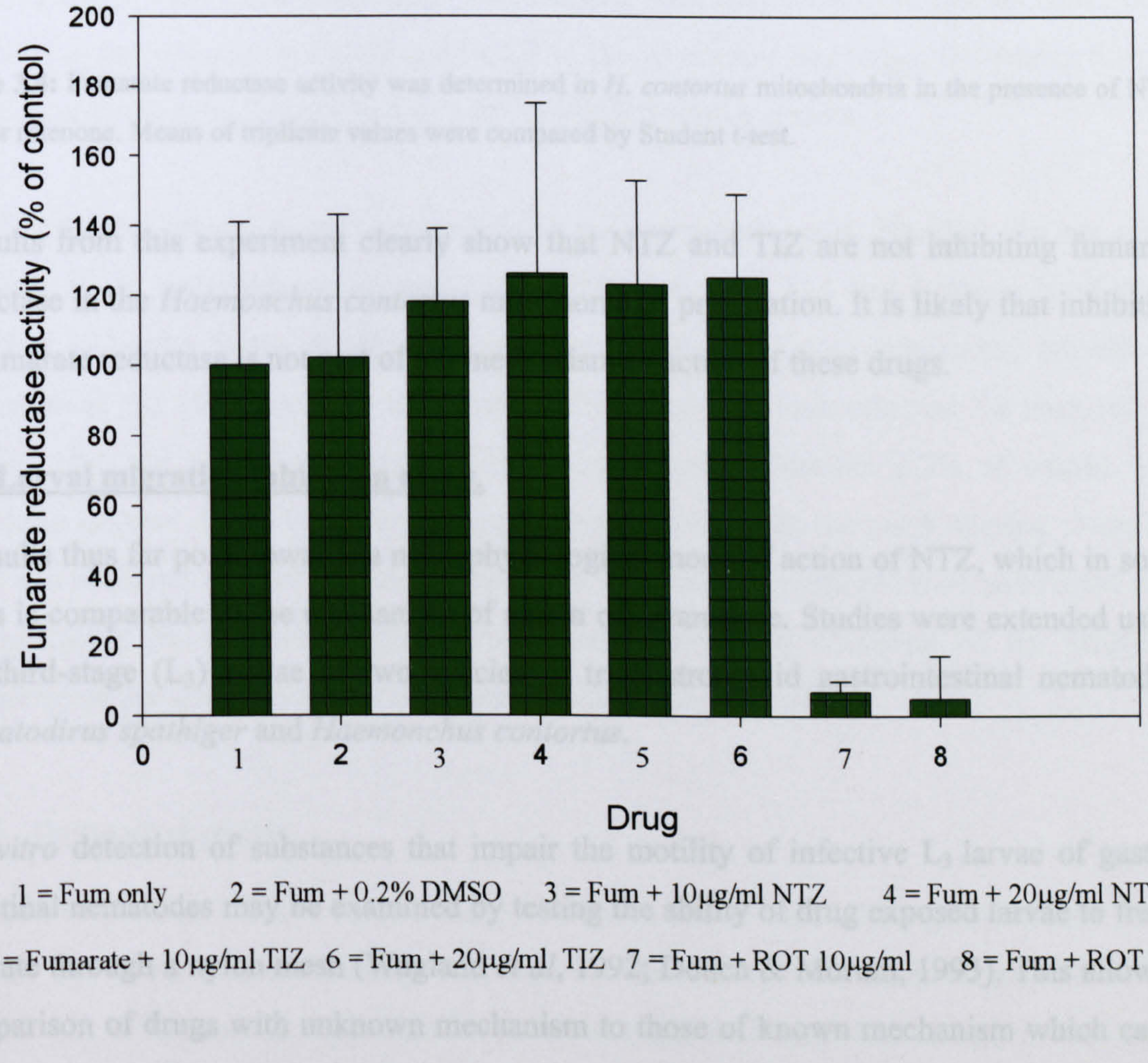
Table 3.3 Effect of NTZ, TIZ, rotenone and DMSO on the fumarate reductase system in *Haemonchus contortus*.

Additions	Rate of NADH oxidation (nmol/min/mg protein) ± s.d.	Percentage of rate of NADH oxidation in presence of fumarate
NADH Hepes + 0.2% DMSO	18.7 ± 2.12	68.9
NADH Hepes + 10µg/ml NTZ	20.6 ± 0.6	76
NADH Hepes + 10µg/ml rotenone	3.9 ± 1.1	14
Fumarate + NADH	27.1 ± 0.5	100
Fumarate + NADH + 0.2% DMSO	27.8 ± 3.8	103

Additions Fumarate rate only	Rate of NADH oxidation (nmol/min/mg protein) ± s.d.	Percentage of fumarate rate
Fumarate + 0.2% DMSO	9.1 ± 3.8	102
Fumarate + 10µg/ml NTZ	10.8 ± 1.9	118
Fumarate + 20µg/ml NTZ	11.5 ± 4.5	126
Fumarate + 10µg/ml TIZ	11.2 ± 2.7	123
Fumarate + 20µg/ml TIZ	11.4 ± 2.4	125
Fumarate + 10µg/ml rotenone	0.7 ± 0.3	7
Fumarate + 20µg/ml rotenone	0.5 ± 1.1	5

Fumarate reductase activity was calculated by subtracting the NADH rate + fumarate from the NADH rate + an equal volume of buffered Hepes (replacing fumarate). The reaction rates are expressed as means ± standard deviation. T tests were carried out using a Student t-test with $P < 0.05$ considered significant (see table 3.4).

Figure 3.5 Change in fumarate reductase activity in the presence of NTZ, TIZ, ROT and DMSO.



1 = Fum only 2 = Fum + 0.2% DMSO 3 = Fum + 10µg/ml NTZ 4 = Fum + 20µg/ml NTZ

5 = Fumarate + 10µg/ml TIZ 6 = Fum + 20µg/ml TIZ 7 = Fum + ROT 10µg/ml 8 = Fum + ROT 20µg/ml

Fig 3.5: Fumarate reductase was measured via NADH oxidation spectrophotometrically at 340nm. Rotenone at 10µg/ml significantly inhibited fumarate reductase ($P < 0.0005$). NTZ, TIZ (10 and 20µg/ml) and DMSO increased fumarate reductase activity. Rate of NADH oxidation in the presence of fumarate was also increased by NTZ, TIZ and DMSO.

Table 3.4 Effect of NTZ, TIZ and ROT on fumarate activity.

Drug rate.	Control	NTZ (10µg/ml)	TIZ (10µg/ml)	Rotenone (10µg/ml)
Mean fumarate reductase activity. nmol/min/mg protein	9.1 ± 3.8	10.8 ± 1.9	11.2 ± 2.7	0.7 ± 0.3
P value.	-	0.3	0.2	0.0004

Table 3.4: Fumarate reductase activity was determined in *H. contortus* mitochondria in the presence of NTZ, TIZ or rotenone. Means of triplicate values were compared by Student t-test.

Results from this experiment clearly show that NTZ and TIZ are not inhibiting fumarate reductase in the *Haemonchus contortus* mitochondrial preparation. It is likely that inhibition of fumarate reductase is not part of the mechanism of action of these drugs.

3.9 Larval migration inhibition assay.

Results thus far point towards a neurophysiological mode of action of NTZ, which in some ways is comparable to the mechanism of action of levamisole. Studies were extended using the third-stage (L_3) larvae of two species of trichostrongylid gastrointestinal nematodes: *Nematodirus spathiger* and *Haemonchus contortus*.

In vitro detection of substances that impair the motility of infective L_3 larvae of gastro-intestinal nematodes may be examined by testing the ability of drug exposed larvae to freely migrate through a nylon mesh (Wagland *et al*, 1992; Douch & Morum, 1993). This allows a comparison of drugs with unknown mechanism to those of known mechanism which cause inhibition of motility. Potential NTZ/TIZ induced inhibition of migration with these parasitic larvae was therefore examined and compared to that of levamisole, a known inhibitor of larval migration (Douch & Morum, 1993).

3.9.1 Motility and migration of *Nematodirus spathiger.*

3.9.2 Sheathed worms.

In preliminary experiments, microscopic examination 2 hours post addition of levamisole, FCCP or CCCP (each at 10μg/ml), showed a clear effect on the motility of *N. spathiger* larvae, characterised by curling and paralysis. Subsequent addition of these paralysed larvae to the migration chambers (containing the same concentration of drug) showed significant inhibition of migration of sheathed worms through the mesh into the well. The inhibition was concentration dependent (figure 3.6) and allowed dose response curves to be calculated, yielding IMg_{50} values (concentration (μM) causing 50% inhibition of migration under these conditions) for levamisole, CCCP and FCCP in the 10 micromolar range. Increasing the pre-incubation times from 2 hours to 4 or 8 hours yielded similar results.

NTZ, TIZ and other Romark compounds at concentrations up to 30μg/ml did not affect motility of *N. spathiger* on microscopic examination after 2, 4 and 8 hours. Neither did addition of larvae to the drugged migration chambers post drug-incubation, prevent the migration of the larvae through the mesh. Mebendazole, niclosamide and the drug solvent DMSO also had no effect. However with these negative controls 2-3% of worms were unable to migrate due to becoming trapped owing to creases in the mesh causing clumping. This was observed also for Romark compounds, and these percentages were subtracted from the positives. No negative control drug induced increased clumping of the larvae as compared to the small amount of clumping which occurs naturally.

3.9.3 Exsheathed worms.

In case the lack of effect of some compounds was due to lack of drug penetration of the sheath, the larval migration assay was repeated as above, with exsheathment of worms carried out using sodium hypochlorite (see materials and methods). IMg_{50}'s were again calculated using Sigmaplot 2000 (Figure 3.6).

Fig 3.6 Determination of inhibition of migration of exsheathed *N. spathiger* by levamisole, FCCP and CCCP.

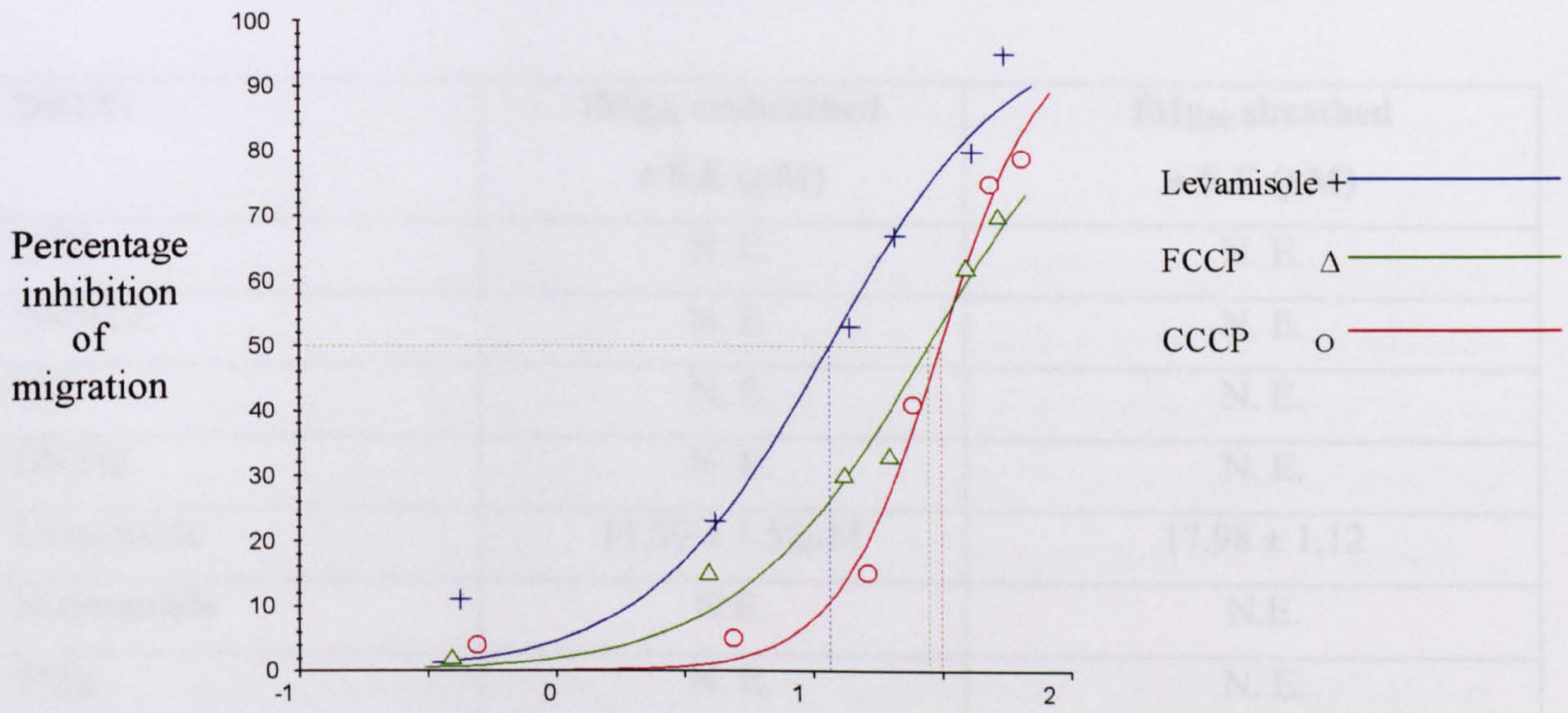


Fig 3.6: Exsheathed *N. spathiger* larvae were exposed to levamisole (+), FCCP (Δ) and CCCP (o) at varying concentrations for 2 hours and ability to migrate through a nylon mesh quantitated in comparison to solvent-tested controls. Data were analysed using Sigmaplot 2000 to obtain IMg_{50} values.

Levamisole, FCCP and CCCP inhibited migration of exsheathed larvae more potently than sheathed larvae, in accordance with the findings of Douch & Morum, 1993. The Romark compounds, however, still showed no inhibitory activity. The results are summarised in table 3.5.

Table 3.5 Summary of effect of drugs on *N. spathiger* L3 larval migration.

DRUG	IMg_{50} exsheathed ± S.E (μM)	IMg_{50} sheathed ± S.E (μM)
NTZ	N. E.	N. E.
DNNTZ	N. E.	N. E.
TIZ	N. E.	N. E.
DNTIZ	N. E.	N. E.
Levamisole	11.50 ± 1.55μM	17.98 ± 1.12
Niclosamide	N.E.	N.E.
TIZg	N. E.	N. E.
BZNT	N. E.	N. E.
CCCP	31.04 ± 1.78μM	39.86 ± 1.32μM
FCCP	27.89 ± 2.53μM	37.54 ± 2.01μM
MEB	N.E.	N.E.

IMg_{50} = Concentration of drug which resulted in inhibition of migration through a nylon mesh in 50% of the worm population.

N. E. = No Effect: Inhibition of migration not detected up to 30μg/ml.

3.9.4. *Haemonchus contortus.*

The inhibition of migration of *H. contortus* was also investigated. Incubation with NTZ, TIZ and other Romark compounds (for 2, 4 or 8 hours) had no inhibitory effect on the migration of *H. contortus* larvae whether sheathed or exsheathed, and did not cause paralysis. This is interesting owing to NTZ (10μg/ml) causing paralysis in 100% adult *H.*

contortus within 3 hours. Levamisole caused both paralysis and migratory inhibition yielding IMg_{50} values of 5.7 ± 0.7μM and 3.2 ± 0.5μM for sheathed and exsheathed larvae respectively. These values are similar to those obtained by Douch & Morum, (1993). The uncouplers FCCP and CCCP again caused paralysis and hence inhibition of migratory movement of *H. contortus* and IMg_{50}'s for FCCP and CCCP were 29.3 ± 1.3μM and 24.0 ± 0.9μM for sheathed larvae and 28.07 ± 1.1μM and 23.22 ± 0.7μM for exsheathed larvae.

3.10 Assay of nitroreductase activity in *C. elegans* with nitazoxanide and tizoxanide.

In anaerobic protozoa, NTZ is thought to be reductively activated to produce a toxic product (Adagu *et al*, 2002). Nitroreductases of sufficiently low redox potential are occasionally found in microaerophilic or facultatively anaerobic species. A nitroreductase for *E. cloacae* previously shown by Bryant & DeLuca, (1991) (see Appendix V) shows sequence homology to a protein of *C. elegans*. Therefore, the reduction of NTZ by *C. elegans* was investigated.

Since nitroreductases for different species may be linked to either NADPH or NADH, they were tested separately as electron donors to NTZ/TIZ and nitrofurazone (NF). In the presence of *C. elegans* clarified homogenate (0.1-0.3mg/ml protein), no change in NTZ/TIZ absorbance at 412nm or NF absorbance at 375nm was seen in the presence of NADH. With NADPH a decrease in absorbance was seen, however this was also observed in the solvent (DMSO) control. Therefore no evidence for a nitroreductase capable of reducing NTZ/TIZ was seen in extracts of *C. elegans* obtained under conditions in which NTZ/TIZ cause paralysis. It remains possible that the putative nitroreductase is expressed only during anaerobic/microaerophilic growth conditions of *C. elegans*.

3.11 Examination of effect of NTZ on ATP levels in *C. elegans.*

Since the protonophores FCCP and CCCP affected *C. elegans* motility, the action of NTZ/TIZ could be due to uncoupling of oxidative phosphorylation as well as nicotinic activity. Therefore the effects of NTZ/TIZ on ATP levels in *C. elegans* were examined. A sensitive luminescence assay was used incorporating the luciferin/luciferase enzyme system, modified from that previously used by Ronner *et al*, (1999) and Ford *et al*, (1996).

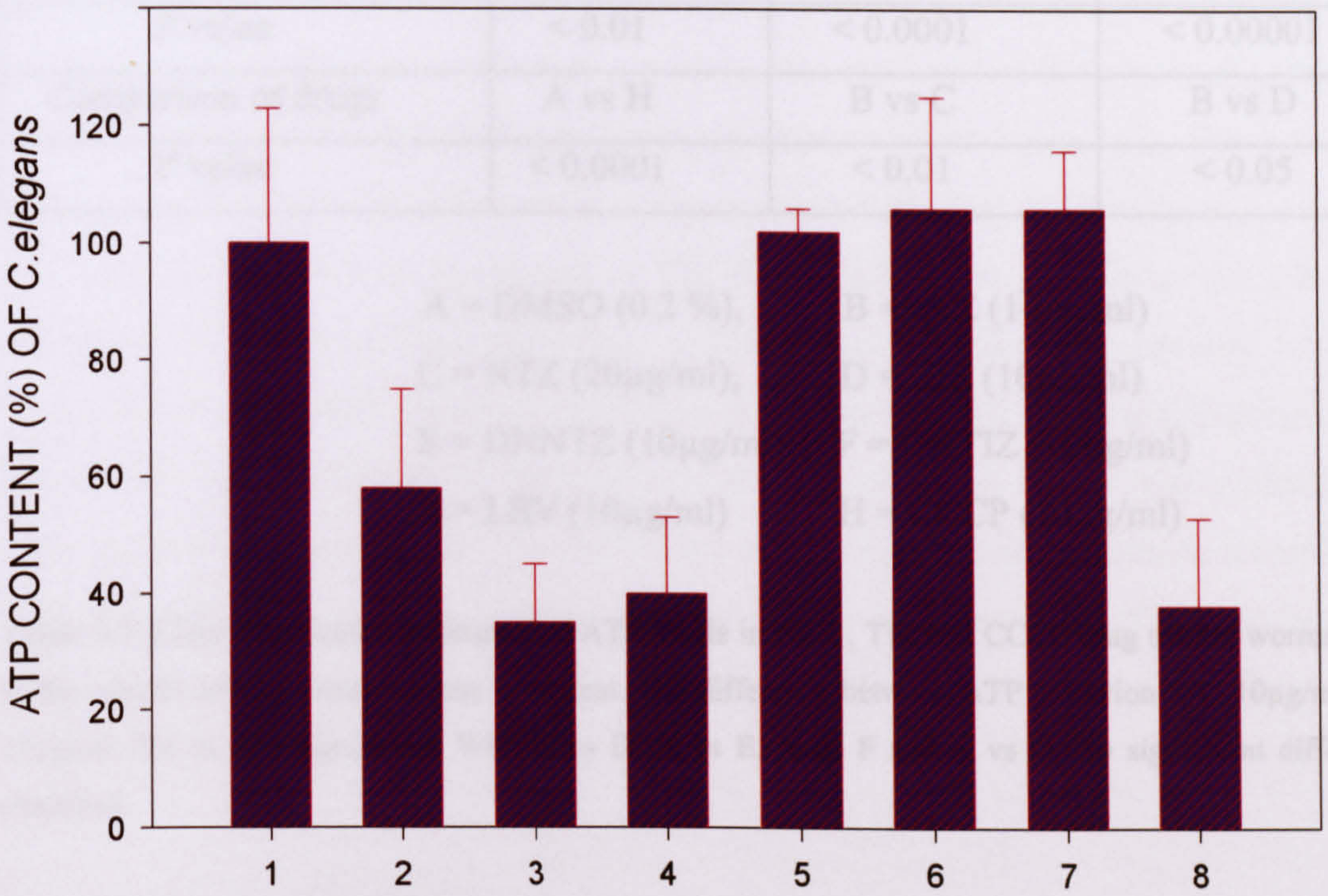
Incubation of *C. elegans* with NTZ and TIZ at 10μg/ml for one hour at ambient temperature, caused a substantial decrease in ATP concentration, with a further decrease at 20μg/ml (Table 3.6). CCCP (10μg/ml) similarly caused a decrease in ATP concentration. The corresponding denitro-compounds and levamisole had no effect on ATP levels. The solvent DMSO at the concentration present when drugs are in the system had no effect on ATP levels which remained comparable to levels found in worms direct from axenic media.

Table 3.6 Effect of NTZ/TIZ on ATP levels in *C. elegans.*

Treatment	DMSO (A) 0.2 %	NTZ (B) 10μg/ml	NTZ (C) 20μg/ml	TIZ (D) 10μg/ml
Mean [ATP] ± s.d. nmol mg protein^{-1}.	4.8 ± 1.1	2.8 ± 0.8	1.7 ± 0.5	1.9 ± 0.6
Treatment	DNNTZ (E) 10μg/ml	DNTIZ (F) 10μg/ml	LEV (G) 10μg/ml	CCCP (H) 10μg/ml
Mean [ATP] ± s.d. nmol mg protein^{-1}.	4.9 ± 0.2	5.1 ± 0.9	5.1 ± 0.5	1.8 ± 0.7

Table 3.6: Mean [ATP] was calculated for each drug and compared to DMSO control. The above table shows the typical values of the drug effects on ATP levels in nmol mg protein^{-1}. The data for several experiments were hence incorporated and analysed in figure 3.7. All drugs used showed no reduction in ATP levels when tested with the standard only.

Figure 3.7 Effect of NTZ/TIZ on ATP content of *C. elegans*.



1 = 0.2 % DMS0 2 = 10µg/ml NTZ 3 = 20µg/ml NTZ 4 = 10µg/ml TIZ

5 = 10µg/ml DNNTZ 6 = 10µg/ml DNTIZ 7= 10µg/ml LEV 8 = 10µg/ml CCCP.

Fig 3.7: *C. elegans* were incubated for 1 hour at room temperature with NTZ (10 or 20µg/ml), TIZ (10µg/ml), DNNTZ (10µg/ml), DNNTZ (10µg/ml), LEV (10µg/ml), CCCP (10µg/ml) or DMSO (0.2%). After total solubilising in alkali, ATP was determined as described in the methods. All assays were carried out in triplicate and similar results were obtained in separate experiments. Significant differences between values were calculated using a Student t-test (see table 3.7).

Table 3.7 Significance testing of ATP levels in drug exposed *C. elegans.*

Comparison of drugs	A vs B	A vs C	A vs D
P value	< 0.01	< 0.0001	< 0.00001
Comparison of drugs	A vs H	B vs C	B vs D
P value	< 0.0001	< 0.01	< 0.05

A = DMSO (0.2 %), B =NTZ (10μg/ml)
C = NTZ (20μg/ml), D = TIZ (10μg/ml)
E = DNNTZ (10μg/ml) F = DNTIZ (10μg/ml)
G = LEV (10μg/ml) H = CCCP (10μg/ml)

Table 3.7: Clear significance difference of ATP levels in NTZ , TIZ and CCCP drug treated worms compared to the solvent control treated worms is evident. The difference between ATP reduction with 10μg/ml NTZ and 10μg/ml TIZ is also significant. With C vs D, A vs E, A vs F and A vs G no significant difference was observed.

3.12 Discussion.

The mechanism of action of NTZ, TIZ and other Romark compounds was examined using the free-living nematode *C. elegans* as a model. Surprisingly NTZ and TIZ were found to have an effect on the nicotinic receptor of the worm as well as demonstrating an effect on ATP production supporting their proposed protonophore activity. Studies were extended to the parasitic nematodes *A. suum*, *N. spathiger* and *H. contortus*, however, these nematodes were less susceptible to the Romark compounds. The other Romark compounds DNNTZ, DNTIZ and TIZg showed no activity against any nematodes examined.

3.12.1 Possible NTZ neuromuscular mode of action.

a) Acetylcholinesterase inhibition.

Exposure of NTZ or TIZ (10μg/ml) to *C. elegans* induced paralysis which took approximately 20 minutes to develop, was characterised by jerkiness and was transient. This paralysis was similar (but of slower onset) to that observed with levamisole, but differed markedly from the tonic and flaccid paralysis observed with ivermectin. Since paralysis in worms can be caused by either direct receptor interaction (e.g. by levamisole or pyrantel at the nicotinic receptor or ivermectin at the GABA receptor) (Martin *et al*, 1997), or by inhibition of acetylcholinesterase (e.g. pyrantel and metrifonate), causing build up of acetylcholine and hence paralysis (Hardman & Limbird, 1996), these two mechanisms were examined. With NTZ/TIZ, no inhibition in the hydrolysis of ASCh was evident, whereas clear inhibition of hydrolysis with the known acetylcholinesterase inhibitor, metrifonate was observed. This suggests that if a neuroreceptor-type mechanism is in effect, the two Romark compounds may be acting directly on the nicotinic receptor rather than inhibiting acetylcholinesterase.

b) Direct receptor interaction.

Owing to NTZ-induced paralysis being more similar to that of the nicotinic agonist levamisole than ivermectin, direct receptor interaction was hence studied using muscle preparations from *A. suum*, and known nicotinic antagonists. In neurophysiological investigations with *A. suum* muscle preparation, Ach and levamisole (30μM), clearly demonstrated muscle contraction as previously described by Colquhoun *et al*, (1991), however, NTZ did not display any effect until 100μM was added. At this concentration a small contraction was observed, which became larger on addition of higher concentrations. This suggests that NTZ/TIZ has weak nicotinic agonist properties against *A. suum* compared to levamisole. Interestingly the length of the muscle desensitisation period after NTZ addition was quite long, suggesting that the Romark compound has a high affinity for the nicotinic receptor even though *in vitro* potency is low. (this may be an artifact due to the high drug concentration used). Addition of NTZ up to 600μM, did not abolish the Ach or levamisole induced contraction, indicating that the NTZ/TIZ effect on the worm is not due to

interaction with chloride-channels and is therefore not GABA related. TIZ showed no effect on *A. suum* muscle preparation, but higher concentrations of the drug could not be tested due to its poor solubility.

With pre- and post-treatment of the nicotinic antagonists mecamylamine and pempidine (20μM), the effects of 10μg/ml NTZ/TIZ were temporarily prevented or abolished. This is similar to the prevention of levamisole induced paralysis in susceptible nematodes in the presence of mecamylamine and pempidine (James & Gilles, 1985). Pre- and post-treatment with the muscarinic acetylcholine antagonist, atropine (20μM) showed no prevention or any abolition of NTZ/TIZ induced drug effect. This again suggests that NTZ/TIZ is acting directly on the nicotinic receptor in *C. elegans*.

c) Mutants.

Nicotinic receptor interaction was further examined using the known *C. elegans* levamisole-resistant mutants 1072, 904 and lev1. Neither levamisole nor NTZ/TIZ affected the motility of these mutants, but did cause paralysis in the GABA-resistant *C. elegans* mutant, 407 at the same time as paralysis was observed with the wild-type. This suggests NTZ is acting specifically at the nicotinic receptor in the worm. However, the paralysis or lack of paralysis in the GABA mutant induced by levamisole or NTZ/TIZ was difficult to discern as the mutant already had severe motility disfunction and the drug-induced changes were very subtle.

d) BTX.

Unlike with trematodes, examination of rhBTX localisation was of little use with *C. elegans*. Due to BTX being a large peptide (fw 8500), it could not be absorbed across the cuticle. It appears though that BTX was being ingested since fluorescence was observed in the pharyngeal area.

3.12.2 NTZ as a protonophore.

a) *C. elegans.*

Examination of NTZ/TIZ structure has suggested that these drugs may have protonophoric activity (D.C.Warhurst, unpublished data, 2000). In order to examine this possible mechanism of action in NTZ/TIZ, the action of the known protonophores FCCP and CCCP on *C. elegans* were compared with NTZ and TIZ. However, preliminary experiments showed FCCP and CCCP at 10μg/ml to cause a more rapid and tonic paralysis in the worm than that observed with NTZ. Also NTZ or TIZ induced paralysis was transient, with worms able to make a recovery, whereas worms exposed to FCCP and CCCP made no recovery. This lack of recovery in *C. elegans* following FCCP and CCCP would be expected from a protonophore and recovery in NTZ/TIZ exposed worms suggests a different mode of action. However non-lethal protonophoric activity may occur due to either NTZ being metabolised and hence detoxified by the worm or the worm ceasing drug ingestion. NTZ may also be more rapidly excreted from *C. elegans* than FCCP and CCCP. Niclosamide, though previously shown to have activity against the nematode *Angiostrongylus cantonensis in vitro* (Sano *et al*, 1982) did not affect *C. elegans*. This may be due to metabolism of the drug or a delayed toxic effect.

In the larval migration study, both FCCP and CCCP demonstrated an inhibitory effect that was not seen with any Romark compound. According to Fry & Jenkins, (1984) the free-living L_3 larvae of the trichostrongylid *Nippostrongylus brasiliensis* are susceptible to uncoupling by protonophores. Thus inhibition of larval migration caused by possible oxidative phosphorylation inhibition due to FCCP or CCCP is a possibility. NTZ and TIZ could also be able to demonstrate this effect, yet, owing to these Romark compounds having lower log P values than FCCP and CCCP, it is possible that NTZ/TIZ were unable to enter the larvae by trans-cuticular absorption. This is also in accordance with Fry & Jenkins, (1984) where not all electron transport inhibitors were able to penetrate nematode cuticles. Therefore potential lack of drug absorption across the nematode cuticle did not allow a satisfactory comparison between NTZ/TIZ and FCCP/CCCP in this study.

NTZ and TIZ however, at motility-inhibiting concentrations, significantly lowered ATP concentrations in *C. elegans*, as did FCCP and CCCP, while levamisole at 10μg/ml did not. This reduction of ATP was possibly due to NTZ/TIZ acting as uncouplers of oxidative phosphorylation, a mechanism supported by the similar results observed with FCCP and CCCP. However the difference in paralysis between NTZ and FCCP/CCCP suggests a differing mechanism or the presence of a secondary mechanism with NTZ. Another possibility is that FCCP and CCCP may be more potent than NTZ/TIZ.

b) *A. suum.*

Examination of the possible protonophoric effect of NTZ/TIZ was tested using oxygen uptake in adult *A. suum* mitochondria. However, though oxygen uptake was observed, no increase in rate was observed with addition of succinate or ADP. Also, addition of NTZ/TIZ, FCCP or CCCP failed to have any effect on the rate of respiration. This may be due to a couple of reasons. Firstly after *A. suum* matures to the L_3 stage it becomes increasingly anaerobic and the specific activity of fumarate reductase increases remarkably (Takamiya *et al*, 1993). Thus adult *Ascaris* mitochondrial electron transfer proceeds through an NADH-linked reduction of fumarate resulting in the formation of succinate (Köhler & Bachmann, 1978; Köhler & Bachmann, 1980). Any effect of NTZ, TIZ and other uncouplers therefore would not necessarily be detected through changes in oxygen uptake because of the unimportance of this process to the worm in its anaerobic environment. This is further supported in the literature, where Cheah (1976) showed that malate and succinate-supported oxidase activities in *A. suum* were found to be insensitive to antimycin A and cyanide compounds.

3.12.3 NTZ mode of entry.

The mode of entry of NTZ into nematodes is not known. However, owing to there being a difference in speed of onset of paralysis for NTZ/TIZ compared to levamisole, FCCP and CCCP, a few points can be raised. On addition of 10μg/ml NTZ and TIZ, paralysis took

approximately 20 minutes to occur, a time significantly different to the rapid >5 ≤10 minutes onset of paralysis with 10μg/ml levamisole and > 3 ≤5 minutes for 10μg/ml FCCP and CCCP. This suggests a different drug potency or delayed mechanism of drug entry into the worm. Delayed entry is supported by the calculated partition coefficient (log P) for NTZ and TIZ (1.8 and 2.0) being lower than that of levamisole (2.8), and FCCP (3.8) showing that levamisole and FCCP are more lipophilic, and therefore probably more readily able to cross the nematode cuticle, hence producing a faster drug effect. Another possibility for the delayed paralytic response with NTZ/TIZ is that rather than the drug crossing the cuticle, *C. elegans* is ingesting the drug. Experiments using rhBTX, a weak agonist of *A. suum*, nicotinic receptors (Walker *et al*, 1992) show that the *C. elegans* swallow the rhBTX because a large amount of fluorescence is visible in the pharynx. The possible need for drug ingestion for NTZ and TIZ but not with levamisole is further supported by the larval migration assay, where with sheathed *Haemonchus contortus* and *Nematodirus spathiger* L_3 larvae, clear paralytic effects were observed with levamisole producing IMg_{50}'s of 17.98μM and 5.65μM respectively. Effects however, were not seen with NTZ and TIZ.(and hence no inhibition of migration) at double the concentration for 6 times the time to cause 100% paralysis in *C. elegans*. Owing to the trichstrongylid L_3 larvae relying on food reserves and not feeding until they find their position in host (Vanden Bossche *et* al, 1985), it is likely that NTZ and TIZ are not ingested by these larvae, hence the reason no drug activity is observed. This suggestion that the Romark compounds are not able to pass through the cuticle and hence affect the worms appears to be supported. Cuticle importance was further highlighted where the IMg_{50} values for levamisole were less with exsheathed worms, being 11.5μM and 3.2μM for *N. spathiger* and *H. contortus* respectively, showing that sheath plus cuticle may hinder drug entry to a greater extent.

Alternatively to lack of cuticular absorption, NTZ/TIZ may simply have too low an *in vitro* potency (dissociation constant) compared to levamisole to be effective in these species. This was observed in *Ascaris suum* where *in vitro* addition of 600μM NTZ showed a much lower effect compared to 30μM levamisole. This difference of *in vitro* potencies with *C. elegans* has already been observed using pyrantel and morantel. The *in vivo* potencies of pyrantel, morantel and levamisole against parasitic nematodes are very similar (Geary, 1999).

However, when these drugs are used *in vitro* with *C. elegans*, morantel and pyrantel are fifty and one hundred fold less potent than levamisole respectively (Geary, 1999). This may explain why the paralysis with levamisole was more rapid in *C. elegans* than with NTZ, and why levamisole drug activity with *A. suum* was also higher.

3.12.4 Inhibition of fumarate reductase.

From results thus far, a neurophysiological action on nematode nicotinic receptors similar to that of levamisole looked likely. However, levamisole also inhibits fumarate reductase in *H. contortus* (Köhler, *et* al, 1978) and Kuramochi *et al*, 1995 previously proposed that this may be part of the NTZ/TIZ drug mechanism. Therefore, studies with *H. contortus* were carried out to examine potential NTZ/TIZ inhibition of fumarate reductase, However, these drugs did not inhibit fumarate reductase activity in *H. contortus*.

3.12.5 Possible reduction of NTZ as a mode of action.

Another possible mechanism of action of NTZ/TIZ is reductive activation as in protozoa and bacteria (Sisson *et al*, 2002). A nitroreductase in bacteria was found in 1991 (Bryant & DeLuca) which is similar to sequences from *C. elegans* (appendix V). Studies were therefore carried out to investigate if NTZ/TIZ induced paralysis was due to enzyme induced reduction of the drug. However *C. elegans* extracts did not demonstrate detectable NTZ or NF nitroreductase activity. Therefore NTZ/TIZ induced paralysis in *C. elegans* is unlikely to involve drug reduction. Nitroreductase activity however, could still be important as a mode of action in parasitic worms which are metabolically anaerobic as adults e.g. *A. suum*. Also if *C. elegans* could be grown anaerobically there is a possibility that a nitroreductase mechanism could occur.

3.12.6 Conclusions.

From work with nematodes, the majority of results are consistent with a neurotoxicological effect on the nicotinic receptor of the worm being the primary mechanism of action of

NTZ/TIZ. However inhibition of ATP synthesis presumably due to protonophoric uncoupling also occurs. Difference in drug effect was evident with different species of nematodes with *C. elegans* being more susceptible than *A. suum*, and trichostrongylid larvae remaining unaffected. This may be due to nicotinic receptor structural variation, variable absorption or drug detoxification.

NTZ has been shown to be highly active against a variety of nematodes *in vivo* (Davila-Gutierrez *et al*, 2002; Favennec *et al*, 2003; Juan *et al*, 2002) and has shown comparable cure rates with well established anthelmintics e.g. mebendazole and praziquantel. It remains a possibility therefore, that the *in vitro* potency of NTZ is lower than other drugs hence producing low activity, whereas *in vivo* the drug potency is higher, resulting in increased worm susceptibility. There is also a large genetic variation between different species of nematodes and this could also explain the difference in NTZ potency.

CHAPTER 4 – TREMATODES.

4.1 Introduction.

Though anthelmintic chemotherapy for trematodes is currently satisfactory, the finding of novel compounds is still a necessity. Praziquantel (PRAZ) is the drug of choice for schistosomiasis and other trematode infections e.g. paragonimiasis and fasciolopsiasis (De Silva, 1997), with metrifonate (for *Schistosoma haematobium*), oxamniquine (for *Schistosoma mansoni*) and in some cases mebendazole (for *S. mansoni* and *S. haematobium*) also being used. However, the possibility of resistance developing against PRAZ is a matter of concern and a new antischistosomal drug is needed. Furthermore, PRAZ has only very low activity against *Fasciola hepatica* (De Silva, 1997). Recently, the novel anthelmintic NTZ has shown good activity *in vitro* against *F. hepatica* and has produced a cure rate of 87% in clinical trials (Rossignol *et al*, 1998; Favennec *et al*, 2003). Although NTZ has not shown promise in animal experiments with schistosomes, (Romark Data) preliminary results *in vitro* showed a potent drug effect indicating that these organisms would be useful in examining mechanisms of action *in vitro*. This chapter describes investigations into the mechanisms of effect of nitazoxanide in *Schistosoma mansoni* and *Schistosoma japonicum*. Effects on worm motility, worm morphology, ATP synthesis and nutrient uptake are examined. Drug effects on the five different stages of *Schistosoma mansoni* are also noted and compared.

4.2. Effects of Romark compounds and control drugs in the absence of serum.

4.2.1 Nitazoxanide and tizoxanide: effects on adult *S. mansoni* and *S. japonicum*.

Pairs of freshly isolated *S. mansoni* were placed into 1.5ml of medium 169 (without phenol red), in wells of a 24 well flat-bottomed microplate (Nunc) at 37°C. NTZ or TIZ in DMSO were added at varying concentrations ranging from 0.1 to 30µg/ml and the effects observed using a Reichert–Jung inverted microscope at 20x magnification and ambient temperature. In contrast to the slow and partial effects seen with *C. elegans*, addition of

10μg/ml NTZ or TIZ (33μM and 38μM respectively) caused the worm pair to curl and become paralysed within 2 minutes.

Paralysis was tonic, characterised by rigidity and shrinkage (Figure 4.1c). Within 10 minutes worms were moribund, showing no movement except in the gut. Males were affected somewhat faster .than females, and worm couples became separated within 30 minutes. After 2-3 hours, clear tegumental damage was seen, particularly in the males, characterised by blebbing, granulation, vesiculation and darkening. There was no sign of recovery and all worms were dead at 24 hours. These effects were similar for all concentrations tested, with 0.7μM NTZ and 0.8μM TIZ being the minimum concentration to kill 100% of the worms (though onset of paralysis and worm death was less rapid). At concentrations below these, the onset of sluggishness etc was very slow, taking up to 24 hours to occur. Also, at these lower concentrations the difference in drug effect between males and females was more marked. For males, sluggishness and some paralysis still occurred at 0.3μM, but for females, concentrations below 0.5μM produced little effect. The DMSO solvent controls did not show significant differences in motility or appearance from the untreated controls. Untreated worms in medium 169 remained active for up to 15 days in a 37°C incubator in the presence of 5% CO_2/ 95% air. A comparative NTZ/TIZ effect on four week old juvenile worms was identical to that observed with the seven week old adult worms.

If the medium containing 0.7μM or less NTZ/TIZ was removed after 4 hours, and replaced with fresh medium, a partial recovery of activity occurred, but the worm tegument did not repair. However, at 10μg/ml (33μM and 38μM for NTZ and TIZ respectively) drug concentration, no reversal of toxicity was apparent and 100% worm death was observed after 24 hours.

Similar experiments were carried out using fresh isolates of adult *S. japonicum*. At 10μg/ml the effects of NTZ and TIZ on *S. japonicum* involved curling and paralysis in a similar way to that observed with *S. mansoni*. Again worms became moribund after 10

minutes and 100% mortality was reached within 24 hours. However, due to a lack of availability of *S. japonicum*, the range of concentrations tested was restricted.

4.2.2. Effect of praziquantel on adult *S. mansoni.*

In the same way as above, the control drug PRAZ was added to *S. mansoni*. At 10µg/ml (32µM) PRAZ caused immediate paralysis in 100% of worms. This effect was similar to that seen with NTZ and TIZ except that the observed paralysis was different (see fig 4.1.b), characterised by a flaccid curling, shrinkage and darkening, which compares differently to the rigidity of the curling with NTZ. Again 100% of worms showed total flaccid paralysis after 10 minutes and all were moribund between 10 to 30 minutes. Unlike NTZ and TIZ however, death of the worms could take up to five days at this concentration. At lower concentrations the paralytic effect of PRAZ was the same and just as rapid, with the lowest concentration to kill 100% of worms within 5 days being approximately 0.5µM.

4.2.3. Effect of other Romark compounds with adult *S. mansoni.*

Of the other Romark compounds, DNNTZ, DNTIZ and TIZg had no effect on motility or morphology in adult *S. mansoni*, at concentrations between 0.1µg/ml and 30µg/ml. With BZNT (10µg/ml) (40.1µM), paralysis was observed as with NTZ and TIZ. The observed paralysis was similar in appearance, but took longer to occur than with NTZ and TIZ, however blebbing, granulation and darkening of the tegument were still in evidence. Exposure to BZNT at 10µg/ml did not result in a killing effect with adult worms and all worms showed some movement, though characterised by sluggishness, after 120 hours.

4.2.4 Effect of Romark compounds with schistosomula of *S. mansoni.*

Schistosomula of *S. mansoni* when exposed to 10µg/ml NTZ and TIZ at 37°C became rapidly paralysed but unlike adults, paralysis was characterised by a total lack of motility. Again in the schistosomula, tegument damage was observed and severe blebbing was present at higher concentrations. Unlike with the adults, NTZ and TIZ exposure to schistosomula at lower concentrations (< 1µM), still led to 100% death and no recovery was observed at 0.7µM when removed from drug after 2 hours. With BZNT paralysis was

rapid as with NTZ and TIZ and 0.2μM BZNT killed 100% of exposed schistosomula. This lesser effect observed with adults and strong effect with schistosomula, suggests that BZNT has a slightly different mode of action to that of NTZ and TIZ. The solvent control DMSO had no effect on schistosomula. In medium 169, schistosomula were able to survive at least 7 days in a 37°C incubator in the presence of 5% CO_2/ 95% air.

4.2.5 Effect of praziquantel and niclosamide with schistosomula of *S. mansoni.*

PRAZ and NIC were added to schistosomula in the same way as described for NTZ, in a concentration ranging from 0.1 to 20μg/ml. Where these concentrations of NTZ caused rapid paralysis in 100% of schistosomula, addition of PRAZ at all concentrations showed no effect on worm motility or morphology in a time period of up to 3 hours. After this time however, sluggishness was observed at concentrations above 10μg/ml (32μM) and at 24 hours 100% of schistosomula showed paralysis. Blebbing and tegumental damage however, were not seen. NIC at 10μg/ml (30.6μM) caused rapid paralysis in schistosomula and all worms at this concentration were moribund after 2 hours drug exposure and dead within 24 hours. Addition of 1μg/ml (3.1μM) NIC resulted in a slower onset of paralysis with some worms still alive at 24 hours.

4.2.6 LD_{50}'s of *S. mansoni* with Romark compounds and control drugs (no serum).

LD_{50}'s with adult *S. mansoni*, were calculated for NTZ, TIZ, PRAZ and NIC after 120 hours exposure, using Sigmaplot 2000 in the same way as for *C. elegans*. The LD_{50} of drug effect on schistosomula was difficult to calculate due to the low concentrations and speed in which paralysis occurred (<1μM). However after 120 hours drug exposure, death in adult worms was easier to determine and hence LD_{50}'s could be calculated. All LD_{50}'s were at similar concentrations (see table 4.1) and the concentration difference between that which caused death in 100% of worms and no death was very small.

Table 4.1 LD_{50}'s of *S. mansoni* with Romark compounds and control drugs.

DRUG	LD_{50} ± S. E. μM
Nitazoxanide	0.56 ± 0.02
Tizoxanide	0.63 ± 0.03
Praziquantel	0.32 ± 0.03
Niclosamide	0.43 ± 0.01
Tizoxanide glucuronide	(no effect at 113.5)
Denitro-nitazoxanide	(no effect at 153.2)
Denitro-tizoxanide	(no effect at 137.1)

LD_{50} = Concentration of drug which results in 50% death of adult *S.mansoni* after 120 hrs.

S.E = Standard Error.

4.3. Effect of other drug types with adult *S. mansoni.*

4.3.1 Effect of protonophores.

To examine potential protonophore activity as an antischistosomal mechanism of NTZ and TIZ, the known uncouplers niclosamide, FCCP and CCCP and proguanil were used so as to compare effects on adult schistosomes and schistosomula. The anti-cestodal drug, niclosamide had an immediate paralytic effect on schistosomes, killing schistosomula within 24 hours at 15.3μM and limiting movement at 0.6μM. With adult *Schistosoma mansoni*, niclosamide, FCCP and CCCP all caused paralysis in the worm within 2 minutes at 10μg/ml, and death was observed in 100% of worms after 24 hours. This speed of effect, type of paralysis and 100% mortality in the exposed worms after 24 hours was identical to that of NTZ and TIZ. This suggests a similarity in mode of action of NTZ and TIZ to these known uncouplers. The anti-malarial drug, proguanil (tested as some of the other biguanides have effect on mitochondria (Jalling & Olsen, 1984)), also affected adult *S. mansoni* at 10μg/ml (34.5μM) and caused mortality in 25% of worms after 120 hours. The

type, speed and strength of effect however was different to that observed with the other uncouplers in that 100 % mortality was not reached within 24 hours at 10µg/ml..

4.3.2. Effects of known schistosomicidal drugs on adult *S. mansoni.*

Other schistosomicidal drugs of known mechanism of action were compared with Romark compounds. The acetylcholinesterase inhibitor, metrifonate (MET) at 10µg/ml (38.85µM) caused paralysis in 100% of worms, but unlike with NTZ and TIZ, this paralysis took up to 15 minutes to develop. Furthermore the paralysis was characterised (especially in females) by rigidity without any curling (fig 4.1d). Oxamniquine (OXA) also at 10µg/ml (35.8µM), caused excitation and over activity in adult *S. mansoni* after 15-20 minutes, in agreement with the findings of Foster & Cheetham, (1973); Chavasse *et al*, (1979). However after 24 hours all worms were still alive but were sluggish. No mortality was seen in schistosomes with OXA after 120 hours demonstrating a poor *in vitro* activity of the drug under the conditions used.. This again is in accordance with findings of Foster & Cheetham, (1973), who demonstrated very little *in vitro* drug activity below 80µg/ml (286.4µM). Other drugs to show a schistosomicidal effect were lucanthone (LUC), hycanthone (HYC), niridazole (NIR), RO11 and mebendazole (MEB). At concentrations of 10µg/ml all of these drugs caused paralysis. RO11 (a benzodiazepine derivative) was initially the most effective with paralysis occurring at the same rate as NTZ and all worms becoming moribund within 24 hours (Worms however were still alive after 120 hours). With niridazole and mebendazole, paralysis was slower in occurring taking several hours with regards to mebendazole. After 24 hours all worms exposed to niridazole and mebendazole were moribund. With mebendazole death was observed in 25% of worms after 5 days, however with niridazole, no worm death was observed. This suggests that although NTZ and niridazole are structurally similar, the mechanism of action of these two drugs is different, or niridazole may need to be metabolised. Lucanthone 10µg/ml (29.4µM) initially had no *in vitro* effect on *S. mansoni* as previously observed by Pica-Mattoccia & Cioli, 1986. After 24 hours however, paralysis and discolouration of the worms was observed suggesting that the worms had changed lucanthone to the active metabolite hycanthone. Lucanthone caused 100% mortality in worms by 48 hours. However, hycanthone at 10µg/ml (28.05), like lucanthone initially had no apparent effect on worms. After 48 hours however, sluggishness

became evident followed by paralysis. Males were primarily affected more than females and after 120 hours 50% of males were dead with the other 50% moribund, whilst the females were elongated and slightly paralysed. Hycanthone at 10μg/ml caused tegumental damage and granulation with blebs evident in males, recovery after 120 hours was unlikely. Strangely the effect of lucanthone on the schistosomes was greater than that of hycanthone. Ivermectin 10μg/ml (37.1μM) also had an effect on schistosomes. The effect was initially very slow but resulted in 100% worm mortality after 120 hours characterised by tegumental darkening and disruption.

4.3.3 Assay of Artemether and its derivatives with adult *S. mansoni*.

Artemether and its derivatives e.g. artesunate have previously been shown to have schistosomicidal activity *in vivo* (Utzinger *et al*, 2001) and *in vitro* (Xiao *et al*, 2003). Artemether, artesunate and dihydro-artemisinin (DHA) were assayed as before with adult *S. mansoni* at concentrations of 1-20μg/ml to examine and compare effect with that of NTZ and TIZ and the known schistosomicidals. Artesunate, Artemether and DHA at all concentrations tested, showed no effect on motility, morphology or mortality with 100% of test worms showing normal movement compared to that of the DMSO control after 120 hours. These results were as expected, for Xiao *et al*, 2003 state that haemin is required in the medium in order to release the toxic free radical and hence produce a drug-effect.

Other drugs were also tested for NTZ comparison, including levamisole, thiabendazole, oligomycin, SHAM, antimycin A and nitrofurazone. Of these SHAM and oligomycin had some inhibitory effect on motility of the worms but did not cause mortality. The effect of all these drugs was different to that observed with the known uncouplers and NTZ. Of all drugs tested, the known uncouplers (niclosamide, FCCP and CCCP), NTZ, TIZ and PRAZ showed the most marked effect on the worms, all causing 100% mortality after 120 hours in 10μg/ml concentrations.

From the schistosome drug assay, many of the drugs tested affected schistosome activity, with many varying effects. However, only FCCP, CCCP and niclosamide produced a

similar effect to that of NTZ and TIZ in terms of speed of paralysis, type of paralysis and similarity in worm morphology and mortality.

The results of the tests are summarised in table 4.2 (see below) where effect on motility and mortality of the worms is used to demonstrate the activity of the drug. All drugs were applied at 10µg/ml to adult *S. mansoni* in 24 well plates at 37°C and monitored by microscopy at regular intervals. The following criteria were used to compare the effect on worm activity on drug exposure.

Key to table 4.2.

5	Excitatory behaviour (Overly active)
4	Active (normal)
3	Reduced activity
2	Sluggish
1	Moribund (Minimal body Movement, but gut moving)
0	No movement of body or gut ± worm disintegration. Parentheses show percentage mortality.

TABLE 4.2 Drug effects on adult *S.mansoni* motility/activity over time

Drug 10µg/ml	10min	30min	1hr	2hrs	4hrs	24hrs	48hrs	96hrs	120 hrs
NTZ	1	1	1	1	1	0 (100%)	0 (100%)	0 (100%)	0 (100%)
TIZ	1	1	1	1	1	0 (100%)	0 (100%)	0 (100%)	0 (100%)
BZNT	4	4	4	3	3	2	2	2	2
DNNTZ	4	4	4	4	4	4	4	4	4
TIZg	4	4	4	4	4	4	4	4	4
PRAZ	1	1	1	1	1	1	1	0 (12.5%)	0 (94%)
NIC	1	1	1	1	1	0 (100%)	0 (100%)	0 (100%)	0 (100%)
FCCP	1	1	1	1	1	0 (100%)	0 (100%)	0 (100%)	0 (100%)
CCCP	1	1	1	1	1	0 (100%)	0 (100%)	0 (100%)	0 (100%)
OXA	4	4	5	5	5	2	3	3	3
MET	2	1	1	1	1	1	2	2	2
NIRI	2	2	1	1	1	1	1	1	1
MEB	4	4	4	3	2	1	1	1	0 (25%)
RO11	2	1	1	1	1	1	1	1	1
NITF	4	4	4	4	4	4	4	4	4
LEV	4	4	4	4	4	4	4	4	4
LUC	4	4	4	4	4	1	0 (100%)	0 (100%)	0 (100%)
HYC	4	4	4	4	4	3	2	2	0 (25%)
SHAM	3	3	3	2	2	2	2	2	2
THIA	4	4	4	4	4	4	4	4	4
PROG	4	4	4	4	4	4	2	0 (12.5%)	0 (25%)
OLIG	4	4	4	4	4	3	3	2	2
Iver	4	4	4	4	4	4	3	2	0 (100%)
AntiA	4	4	4	4	4	4	4	4	4
ART	4	4	4	4	4	4	4	4	4
ARTES	4	4	4	4	4	4	4	4	4
DHA	4	4	4	4	4	4	4	4	4
DMSO -ve control	4	4	4	4	4	4	4	4	4
Medium -ve control	4	4	4	4	4	4	4	4	4

Drug abbreviations.

NTZ=Nitazoxanide, TIZ=Tizoxanide, BNZT=2-benzamido-5-nitrothiazole, DNNTZ=Denitro-nitazoxanide, TIZg=Tizoxanide Glucuronide, PRAZ=Praziquantel, NIC=Niclosamide, FCCP= Carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone , CCCP= Carbonylcyanide m-chlorophenylhydrazone, OXA=Oxamniquine, MET=Metrifonate, NIRI=Niridazole, MEB=Mebendazole, RO11 = Methylclonazepam, NITF= Nitrofurazone, LEV=Levamisole, LUC=Lucanthone, HYC=Hycanthone, SHAM= Salicyl hydroxamic acid, THIA=Thiabendazole, PROG=Proguanil, OLIG=Oligomycin, Iver = Ivermectin AntiA=Antimycin A, ART= Artemether, ARTES= Artesunate, DHA= Dihydro-Artemisinin. DMSO= Dimethyl sulfoxide.

Figure 4.1 Difference in paralysis between an unaffected worm (DMSO only) and that of worms exposed to 10µg/ml praziquantel, nitazoxanide and metrifonate.

a)

Figure 4.1a shows an adult male of *S. mansoni* 20 minutes after 0.1% DMSO exposure. No paralysis is in evidence.

b)

Figure 4.1b shows an adult male of *S.mansoni* 20 after 10µg/ml praziquantel exposure. The worms clearly show paralysis characterised by curling. Worms are flaccid.

c)

Figure 4.1c shows paralysis in 2 adult males after 20 minutes exposure to 10µg/ml NTZ. The paralysis is characterised by contraction and shrinkage of the worm. This form of paralysis is identical to that seen with niclosamide, FCCP and CCCP.

d)

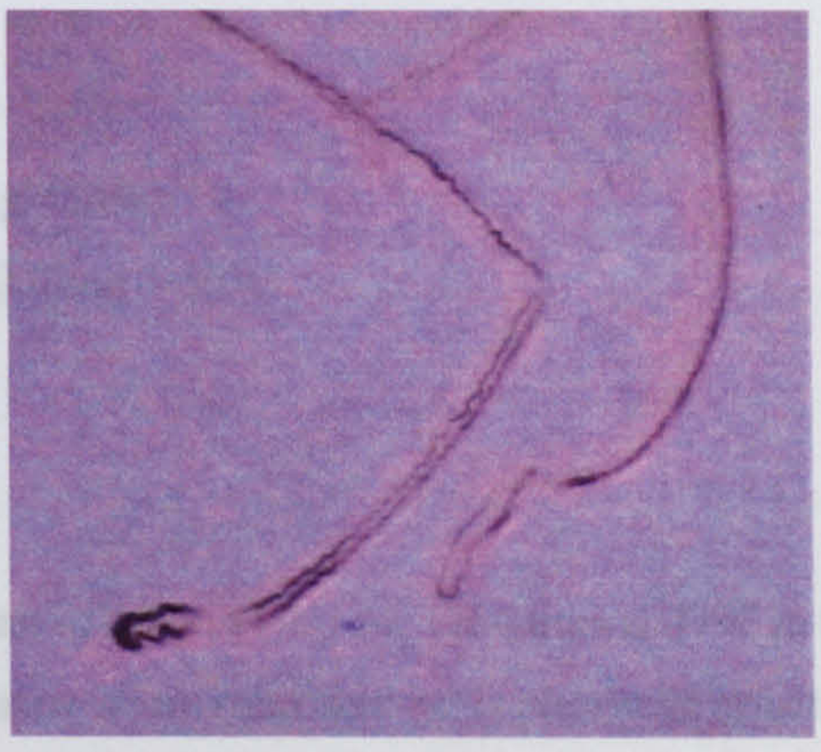
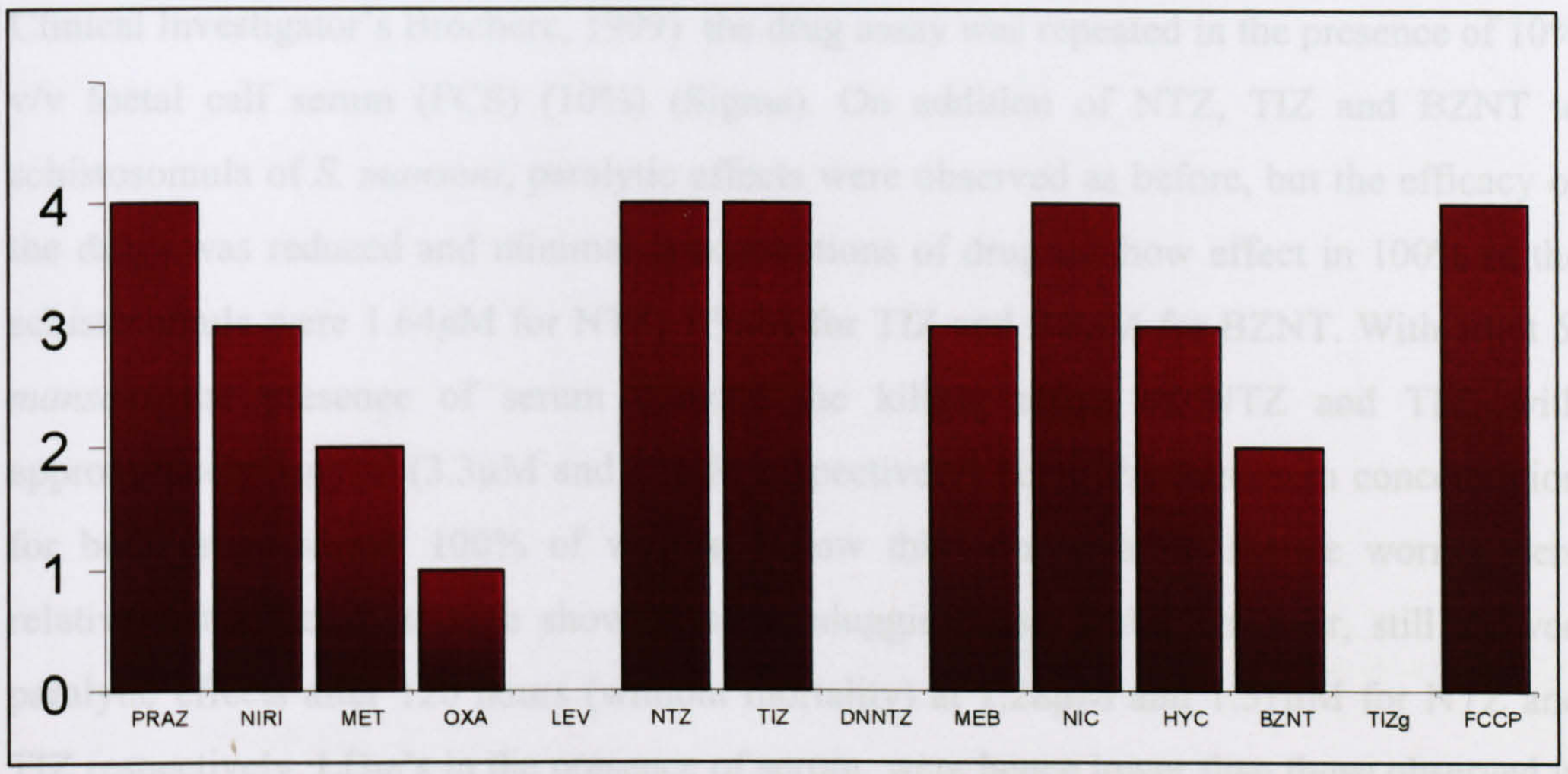


Figure 4.1d shows paralysis in 2 adult females 20 minutes after exposure to 10µg/ml metrifonate. The paralysis is characterised by rigidity in the worm, but instead of contraction the worm elongates.

Fig 4.1: Drugs (10µg/ml) were added to 4 pairs of adult *S.mansoni* in 1.5ml of medium 169. Drug effects observed after 20 minutes incubation were recorded. All pictures are under x40 magnification of a dissecting microscope (Nikon).

Figure 4.2 **Comparison of activity of NTZ/TIZ and other anthelmintics against *Schistosoma mansoni.***



Drug activity is defined as:

0	Not Effective.
1	Slight Effect.
2	Moderate Effect.
3	Good Effect (killing <50%)
4	Very Good Effect (killing >50%)

Fig: 4.2: Four pairs of adult *S. mansoni* were exposed to 10µg/ml of drug i*n vitro* for 120 hours at 37°C in the absence of serum. NTZ and TIZ showed very good activity against worms and activity is clearly as effective as that seen with praziquantel and niclosamide with all drugs killing over 90% of worms after 120 hours. BZNT had a moderate effect as did metrifonate. Both mebendazole and hycanthone showed a good effect, killing 25% of worms. DNNTZ, DNTIZ and TIZg had no effect on schistosomes. The anti-nematodal drug LEV similarly had no effect.

4.4 Effects of drugs on schistosomes in the presence of serum.

4.4.1 Effects of Romark compounds.

Since NTZ and TIZ have a high affinity for albumin (99% binding) (Romark Laboratories Clinical Investigator's Brochure, 1999) the drug assay was repeated in the presence of 10% v/v foetal calf serum (FCS) (10%) (Sigma). On addition of NTZ, TIZ and BZNT to schistosomula of *S. mansoni*, paralytic effects were observed as before, but the efficacy of the drugs was reduced and minimal concentrations of drug to show effect in 100% of the schistosomula were 1.64μM for NTZ, 1.9μM for TIZ and 0.8μM for BZNT. With adult *S. mansoni* the presence of serum reduced the killing effect of NTZ and TIZ, with approximately 1μg/ml (3.3μM and 3.8μM respectively) being the minimum concentration for both drugs to kill 100% of worms. Below this concentration female worms were relatively unaffected (though showing some sluggishness), males however, still showed paralytic effects after 120 hours (without mortality) at 1.28μM and 1.51μM for NTZ and TIZ respectively. LD_{50}'s in the presence of serum, were hence lower than those observed in serum-free medium (see table 4.3). DNNTZ, DNTIZ and TIZg had no effect on adult or juvenile schistosomes in the presence of 10% FCS.

4.4.2 Effects of control drugs.

Control drugs niclosamide and praziquantel were used to compare their activity with the reduced activity of NTZ and TIZ in the presence of serum With adult worms and schistosomula, both PRAZ and NIC, showed no significant difference in their effect and were as active at lower concentrations as they had been without serum. LD_{50} results for adults were calculated and found to be approximately the same as those found when FCS was absent (see table 4.3).

Table 4.3 LD_{50}'s of adult *S. mansoni* with Romark compounds and control drugs in the presence of serum.

DRUG	LD_{50} ± S. E. μM
Nitazoxanide	2.63 ± 0.02
Tizoxanide	2.92 ± 0.02
Praziquantel	0.3 ± 0.01
Niclosamide	0.44 ± 0.02
Tizoxanide glucuronide	(No effect at 113.5)
Denitro-nitazoxanide	(No effect at 153.2)
Denitro-tizoxanide	(No effect at 137.1)

LD_{50} = Concentration of drug which results in death of 50% adult *S.mansoni* after 120 hrs.

S.E = Standard Error.

Differences between the LD_{50} values for NTZ and TIZ in the absence of serum to those LD_{50} values observed with the addition of 10% FCS were calculated using a Student t-test. Values were found to be highly significant with $P < 0.0000001$ for both drugs.

4.5 Schistosome neuromuscular transmission: Receptor investigation.

Since paralysis in nematodes by NTZ was likely due to a specific neuromuscular effect at receptors; effect at schistosome receptors was hence tested using the same nicotinic and muscarinic antagonists as with *C. elegans* and also the specific agonist for schistosome nicotinic receptors α-bungarotoxin (BTX) (Camacho *et al*, 1995).

4.5.1 Receptor antagonists.

As for *C. elegans*, the nicotinic antagonists mecamylamine and pempidine were used to see if they had a reversible or blocking effect on NTZ-induced paralysis in *S. mansoni*. Pempidine (10μM) treatment failed to protect or abolish the NTZ induced effect with adult

S. mansoni. However, pempidine alone also altered the motility of *S. mansoni* causing sluggishness; hence effects of NTZ were difficult to interpret. Mecamylamine did not protect against NTZ-dependent paralysis of adult *S. mansoni* either with co-addition or pre-treatment, neither did it abolish the NTZ/TIZ induced paralysis. This was not unexpected as mecamylamine has previously been found to be an ineffective antagonist of acetylcholine in *S. mansoni* (Day *et al*, 1996). The muscarinic antagonist atropine had no effect on schistosome motility and both pre-treatment and post-treatment did not prevent or abolish paralysis on exposure to 10μg/ml (33μM) NTZ.

Since *S. mansoni* is not readily amenable to genetic manipulation and little of the genome sequence was known, the question of specific effect at nicotinic receptors was addressed using the neurotoxin and nicotinic specific agonist BTX (Tornoe *et al*, 1995; Haughland, 1998).

4.5.2 Effect of BTX.

Addition of 10-40μM BTX showed no effect on motility or viability of adult *S. japonicum* and *S. mansoni*, schistosomula of *S. mansoni* or adult *C. elegans* after 24 hours. BTX likewise, did not show any antagonistic effect and hence demonstrated no drug reversibility in worms paralysed by pre-addition of 10μg/ml NTZ/TIZ. Neither did pre-incubation with BTX for 20 minutes inhibit the paralytic effect of 10μg/ml NTZ/TIZ.

Antagonistic effects with schistosomicidal drugs and specific nicotinic receptor agonists has previously been demonstrated using fluorescent dansyl-choline (DNS-chol) which has specificity for acetylcholine receptors in schistosomes (Hillman *et al*, 1976). In that study the fluorescent binding of DNS-chol was blocked by hycanthone. Also, the fluorescent rhodamine labelled α-bungarotoxin (rhBTX) has been previously used to visualise the nicotinic receptors of *S. haematobium* (Camacho *et al*, 1995). To further examine a possible effect on schistosome nicotinic receptors, rhBTX was used to see if the presence of NTZ/TIZ caused dissociation or failure of rhBTX binding.

4.5.3 NTZ/TIZ: Effect on fluorescent binding of agonists to schistosome receptors.

4.5.3.1 Effect on rhBTX binding.

To test whether NTZ/ TIZ could affect BTX binding, the fluorescent rhBTX was used. RhBTX was able to bind to nicotinic receptors in schistosomula (figure 4.3a) and adult schistosomes (figure 4.4 a) but not *C. elegans* (see earlier). The addition of NTZ or TIZ (10μg/ml) after 20 minute pre-incubation with rhBTX (20μM), resulted in considerable loss of the initial rhBTX fluorescent binding in both schistosomula (figure 4.3 b) and adults (figure 4.4 b). A smoothing of the schistosomulum surface was also observed in the presence of NTZ under differential interference contrast illumination (fig 4.3 b). When worms were exposed to NTZ/TIZ (30μM) for 20 minutes and then rhBTX (20μM) added, failure of rhBTX binding was also observed. At lower concentrations of NTZ (1µg/ml), there was no decrease in binding. Inhibition of rhBTX binding in the presence of NTZ and loss of already bound rhBTX on exposure to NTZ suggests a competition for the nicotinic receptor between rhBTX and NTZ/TIZ. This is consistent with a specific neuromuscular agonist effect of NTZ in the fluke. Untreated schistosomula and adult schistosomes of both species showed no auto-fluorescence under microscope examination.

Figure 4.3a) <u>RhBTX marking of nicotinic receptors in *S. mansoni* schistosomula.</u>

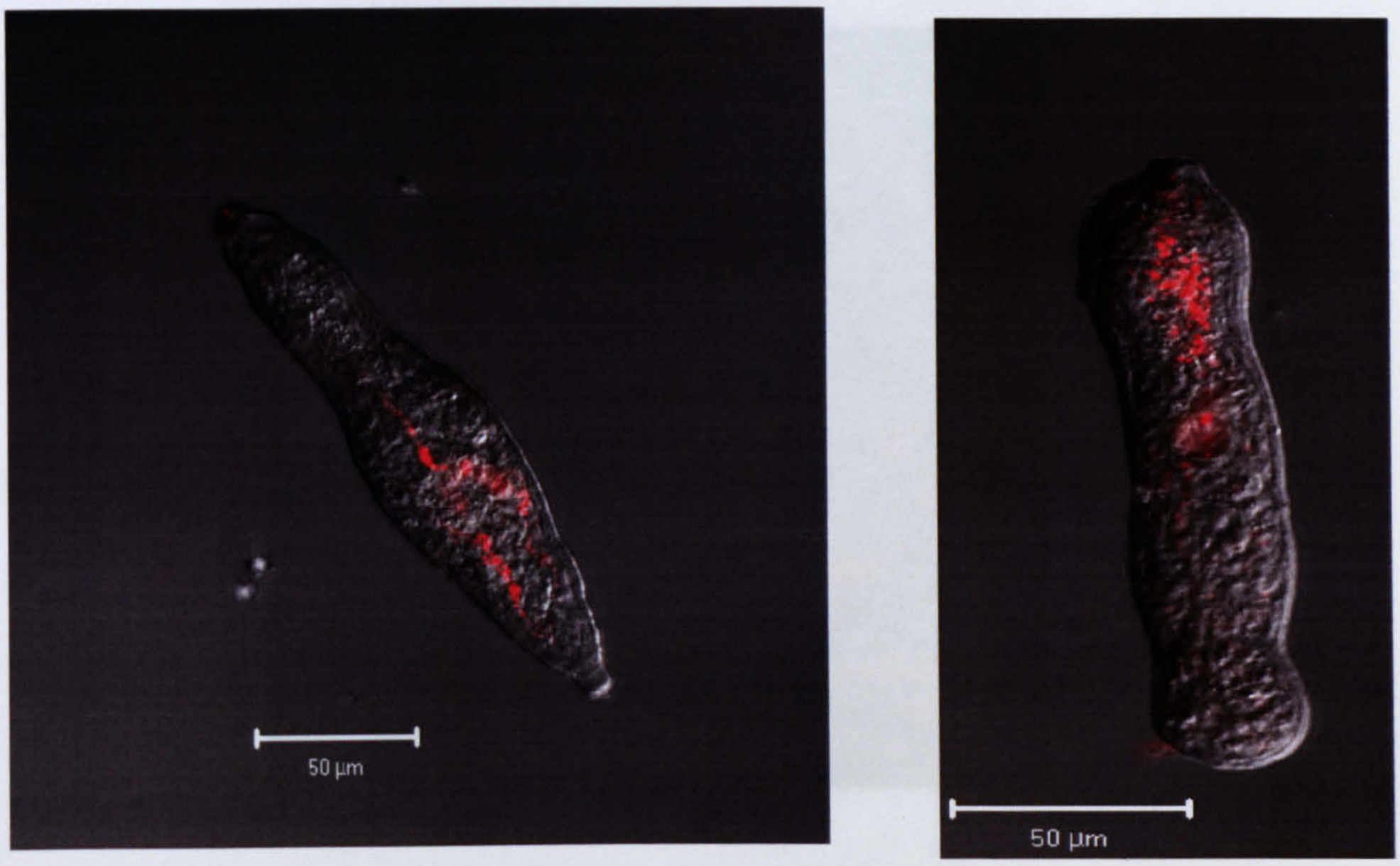


Figure 4.3b) <u>NTZ inhibition of rhBTX binding in *S. mansoni* schistosomula.</u>

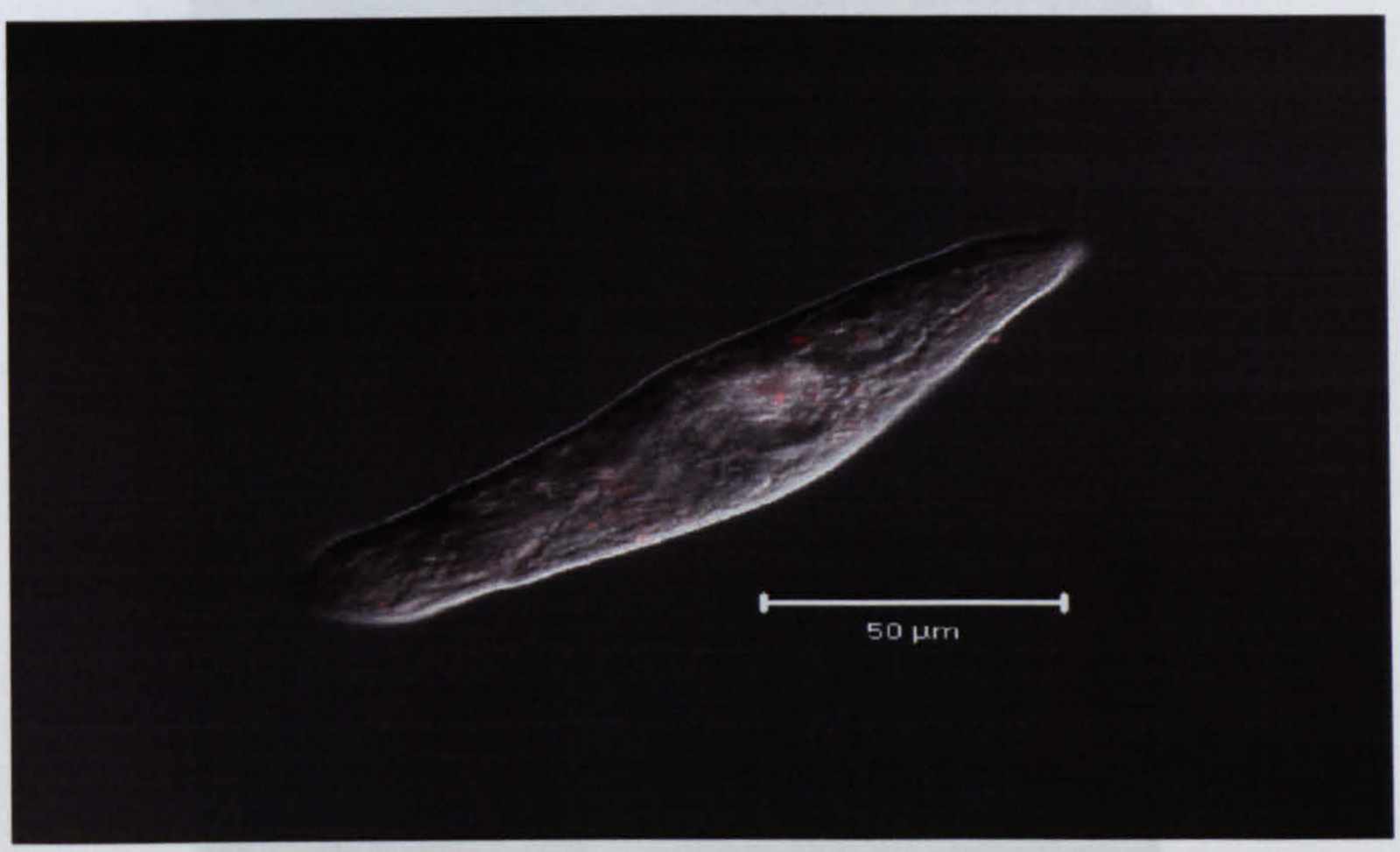


Fig 4.3a: 20µM rhBTX was added to schistosomula and incubation carried out for 2 hours. Clear binding of rhBTX at schistosome nicotinic receptors can be seen In **Fig 4.3 b**) 10µg/ml NTZ was added 20 minutes after rhBTX addition and incubation carried out for a further 2 hours. Typical inhibition of rhBTX binding in the presence of NTZ is shown. Smoothing of the schistosomulum surface is evident.

Figure 4.4:a RhBTX marking of nicotinic receptors in *Schistosoma* adult.

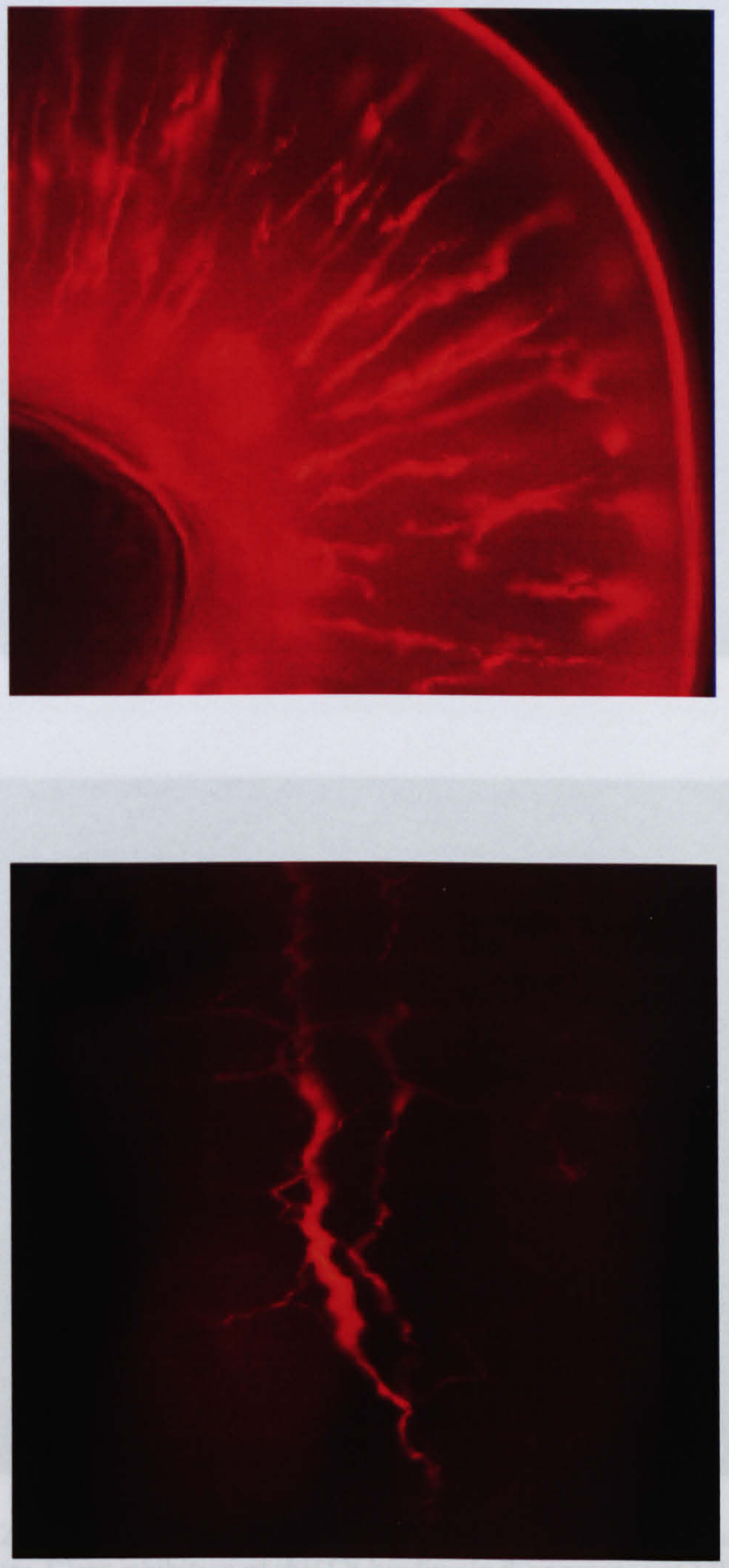


Fig 4.4 a: 20μM rhBTX was added to adult *S.mansoni* (top) and adult *S.japonicum* (bottom) and incubation carried out for 2 hours. Clear fluorescent marking of schistosome nicotinic receptors with rhBTX can be seen.

Figure 4.4 b) NTZ inhibition of rhBTX binding in *Schistosoma* adults.

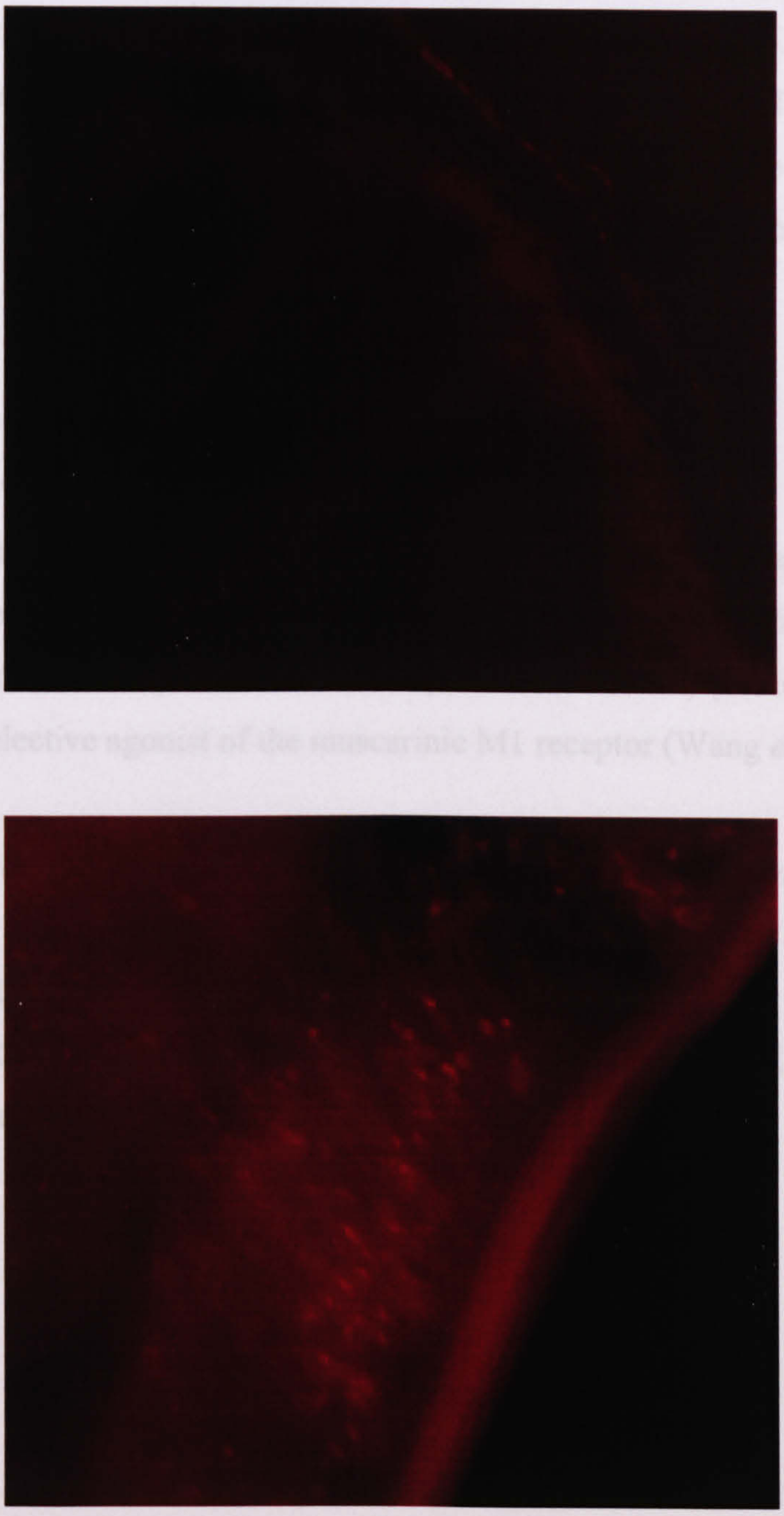


Fig 4.4 b: 10µg/ml NTZ was added 20 minutes after rhBTX addition and incubation carried out for a further 2 hours. Clear inhibition of rhBTX binding in the presence of NTZ can be seen in *S. mansoni* (top) and *S. japonicum* (bottom).

The Romark compounds, DNNTZ and TIZg which do not cause paralysis, used as negative controls showed no inhibition of rhBTX binding in either adult or larval stages of the two schistosome species. BZNT was also used, since it has a severe effect on schistosomes indicating toxicity. However, BZNT did not show inhibition of rhBTX binding in adults, but altered the pattern of fluorescence (not shown) in schistosomula. To test if inhibition was due to a protonophoric effect, CCCP was used. Again however, no inhibition of BTX binding was observed. Other negative controls DMSO and levamisole showed no inhibition of rhBTX binding in schistosomes.

4.5.3.2 Effect on Bodipy pirenzepine binding.

To gain further insight into specificity of NTZ effects at nicotinic receptors, another type of cholinergic receptor was examined: muscarinic. A fluorescent derivative (Bodipy red) of pirenzepine dihydrochloride (excitation 560 nm, emission 569nm) (Molecular Probes) was used, which is a selective agonist of the muscarinic M1 receptor (Wang *et al*, 1994).

When treated with the muscarinic agonist, BoP (6.5μM), *S. mansoni* adults showed widespread dotted fluorescence (figure 4.3a). When NTZ 10μg/ml was added both as pre- and post-treatment to BoP incubated worms, no effect on binding was observed, and the binding pattern actually intensified (figure 4.3b). Control drugs DNNTZ, TIZg and DMSO had no effect on BoP binding.

Figure 4.5 a <u>Bodipy marking of *S. mansoni* adults.</u>

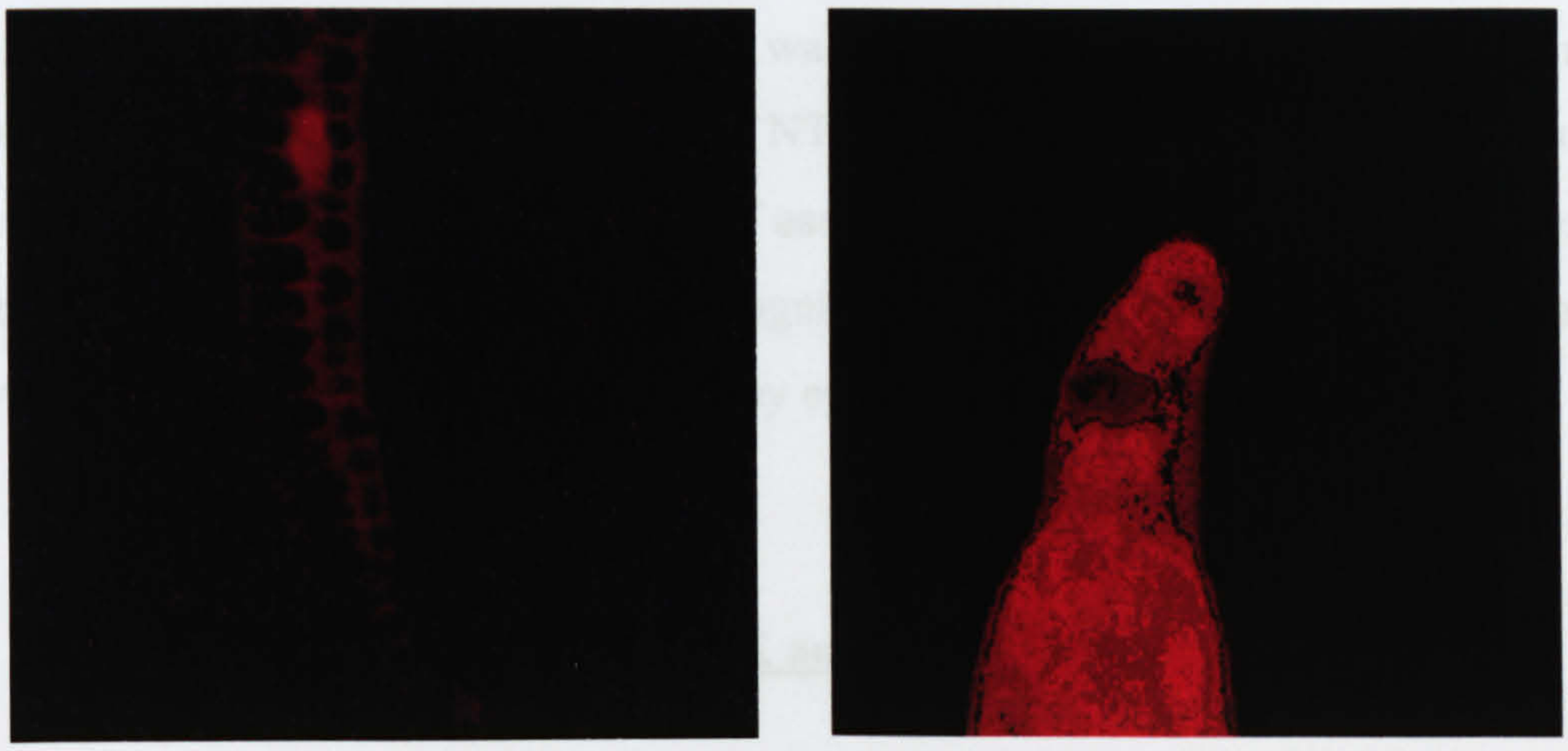


Figure 4.5 b <u>NTZ with Bodipy in *S. mansoni* adult, showing no inhibition.</u>

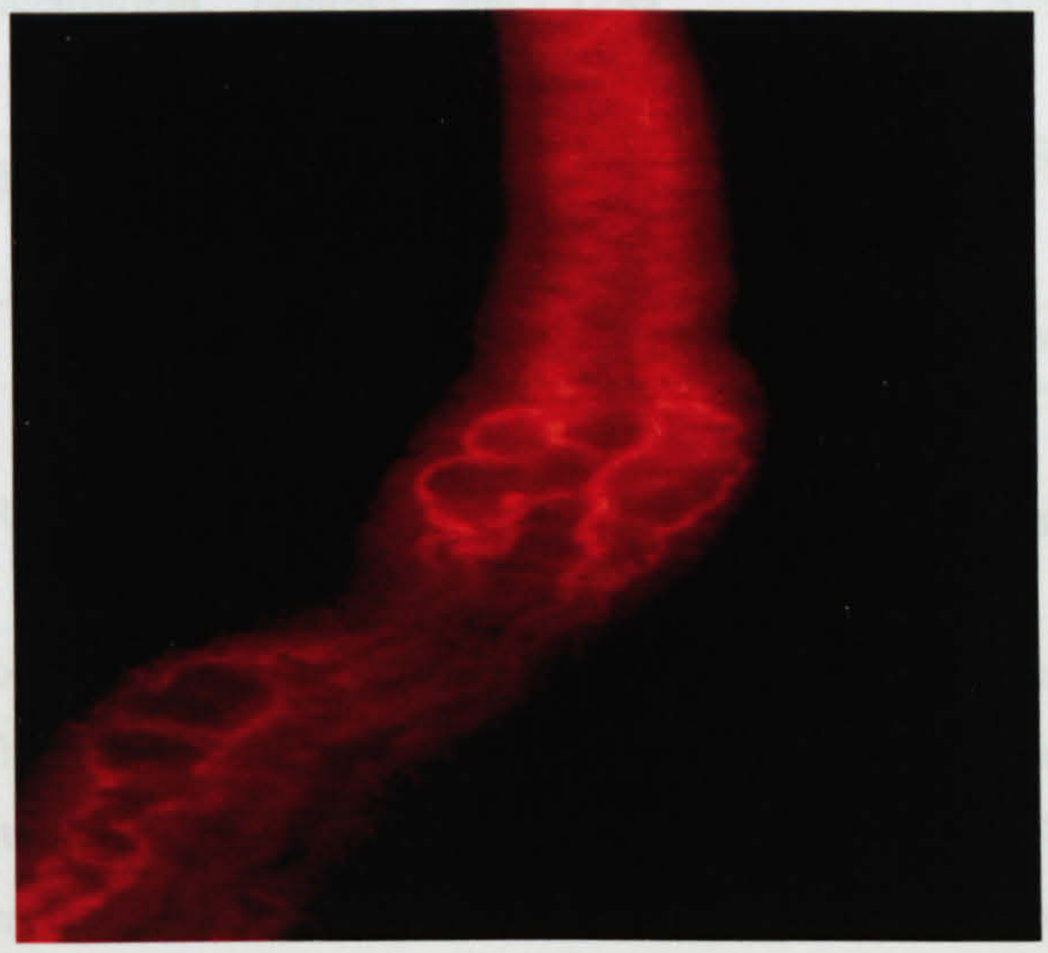


Fig 4.5a: 6.5μM BoP was added to adult *S.mansoni* and incubation carried out for 2 hours. Clear fluorescent marking of schistosome muscarinic receptors with BoP can be seen . In **fig 4.5 b** pre- and post-treatment 10μg/ml NTZ intensifies this binding.

4.6 Test of fluorescent quenching by NTZ.

Inhibition of RhBTX, or BoP fluorescent binding in the presence of NTZ could be due to fluorophore quenching. Possible quenching was determined by examining rhBTX and BoP fluorescence in the presence and absence of NTZ using a Spectra Max Gemini fluorescent plate reader (see table 4.4). A 5µl sample of each of these solutions was then transferred to a clean slide and examined under the 10x magnification of a fluorescent microscope and the relative fluorescence qualitatively assessed by eye.

Table 4.4: Quenching of BTX and BoP fluorescence by NTZ.

Sample	RFU	% quenching
Medium 169	361	n/a
NTZ (33μM)	340	n/a
Tetramethyl α-bungarotoxin (20μM)	2339	n/a
Bodipy Pirenzepine (6.5μM)	695	n/a
Tetramethylα-bungarotoxin (20μM) and NTZ (10μg/ml)	1289	52%
Bodipy Pirenzepine (6.5μM) and NTZ (10μg/ml)	466	33%
Control: DMSO	369	n/a

RFU= Relative Fluorescent Units. n/a = not applicable.

Table 4.4: Effects of NTZ on the fluorescence of rhBTX and BoP was measured in a Spectra Max Gemini fluorescent plate reader using excitation 553nm, emission 577nm for rhBTX and excitation 560nm, emission 569nm for Bodipy with a cut off for both at 565nm. NTZ quenched fluorescence of rhBTX and BoP by 52% and 33% respectively.

On examination of the fluorophores, using the fluorescent microscope, no visible decrease in the amount of rhBTX fluorescence could be determined in the presence of NTZ. Therefore NTZ induced inhibition/dissociation of rhBTX at schistosome nicotinic receptors was due to the presence of drug and not due to quenching of the fluorophores.

4.7 Examination of morphological effect on schistosomes.

4.7.1 SEM of schistosome tegument.

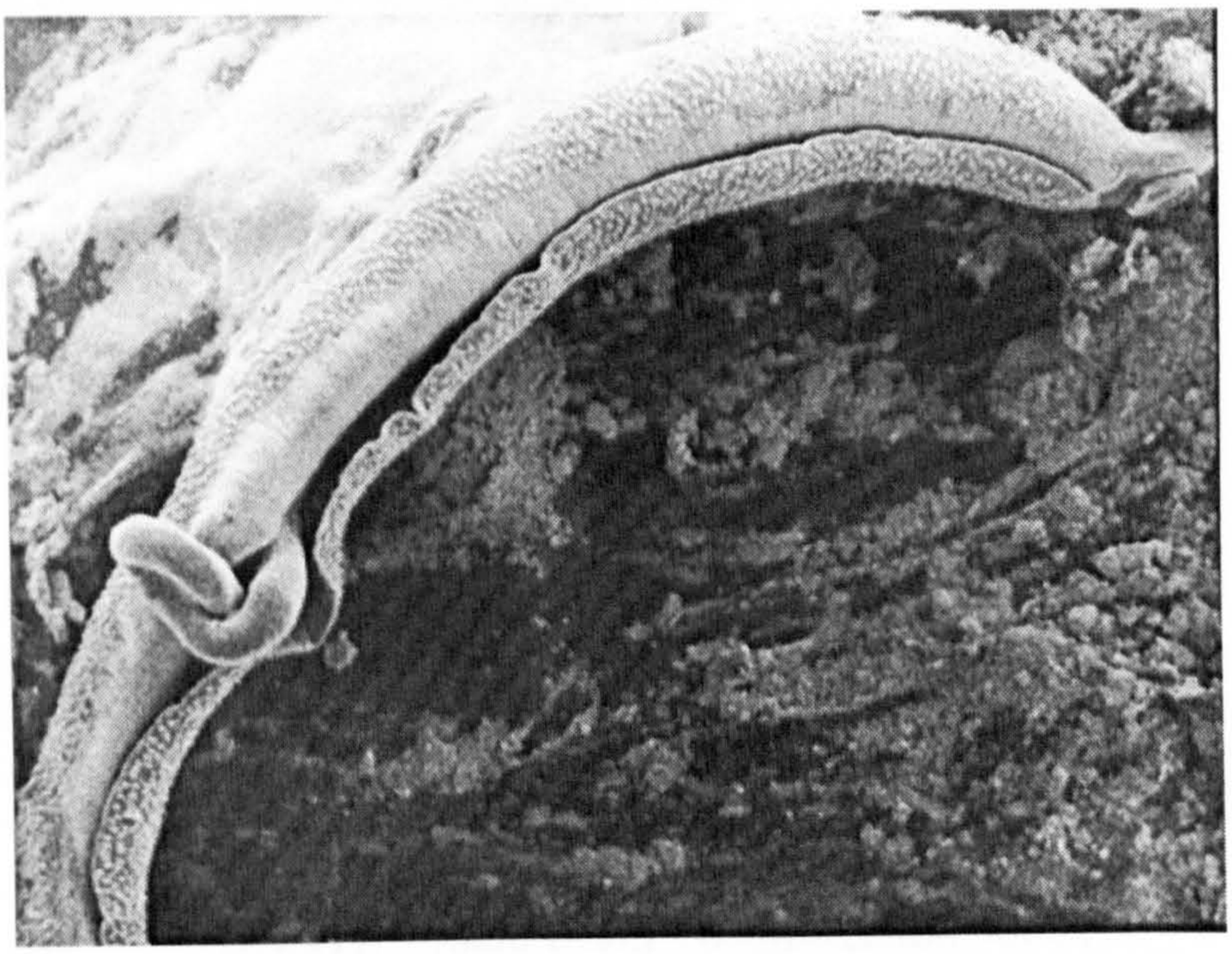
Effects of NTZ on schistosomes have included, blebbing, shrinkage, granulation and darkening of worm tegument. This damage is similar to that observed with PRAZ. Previous work by Apinhasmit *et al*, (1998) and Mansoury, (1997) has demonstrated PRAZ induced tegumental damage with the trematodes *Opisthorchis viverrini* and *S. mansoni* respectively using Scanning Electron Microscopy (SEM). This tegumental damage has been demonstrated in a number of studies to be a major part of drug action e.g. hycanthone with *S. mansoni* and *S. japonicum* (Hillman *et al*, 1977). Therefore to clarify NTZ-induced effects on *S. mansoni* in more detail, the tegument of drug-treated worms was examined by SEM.

Ultrastructural changes of the tegument of adult *Schistosoma mansoni* were investigated after i*n vitro* incubation (without FCS) with 10µg/ml of NTZ, DNNTZ and BZNT for 1 hour. 0.2% DMSO was used as a negative control.

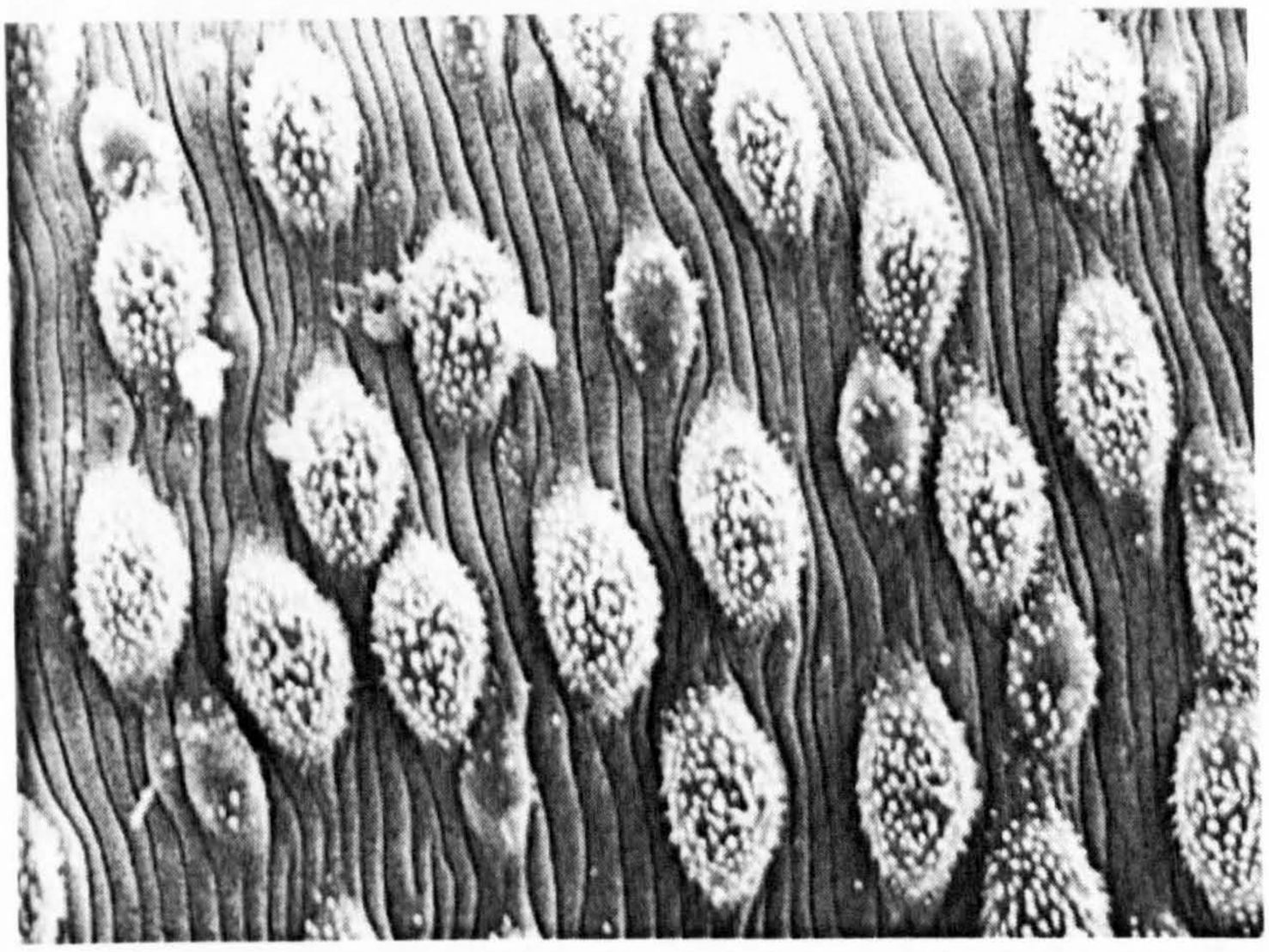
4.7.2 Effect of DNNTZ and DMSO.

The addition of 10µg/ml DNNTZ had no effect on schistosome tegument, with no blebbing, desquamation or formation of lesions evident. This is similar to the DMSO control where tegument disruption was also not observed (Figure 4.6). Addition of either DNNTZ or the drug diluent DMSO (0.2%) had no effect on worm motility and did not cause worm pairs to separate.

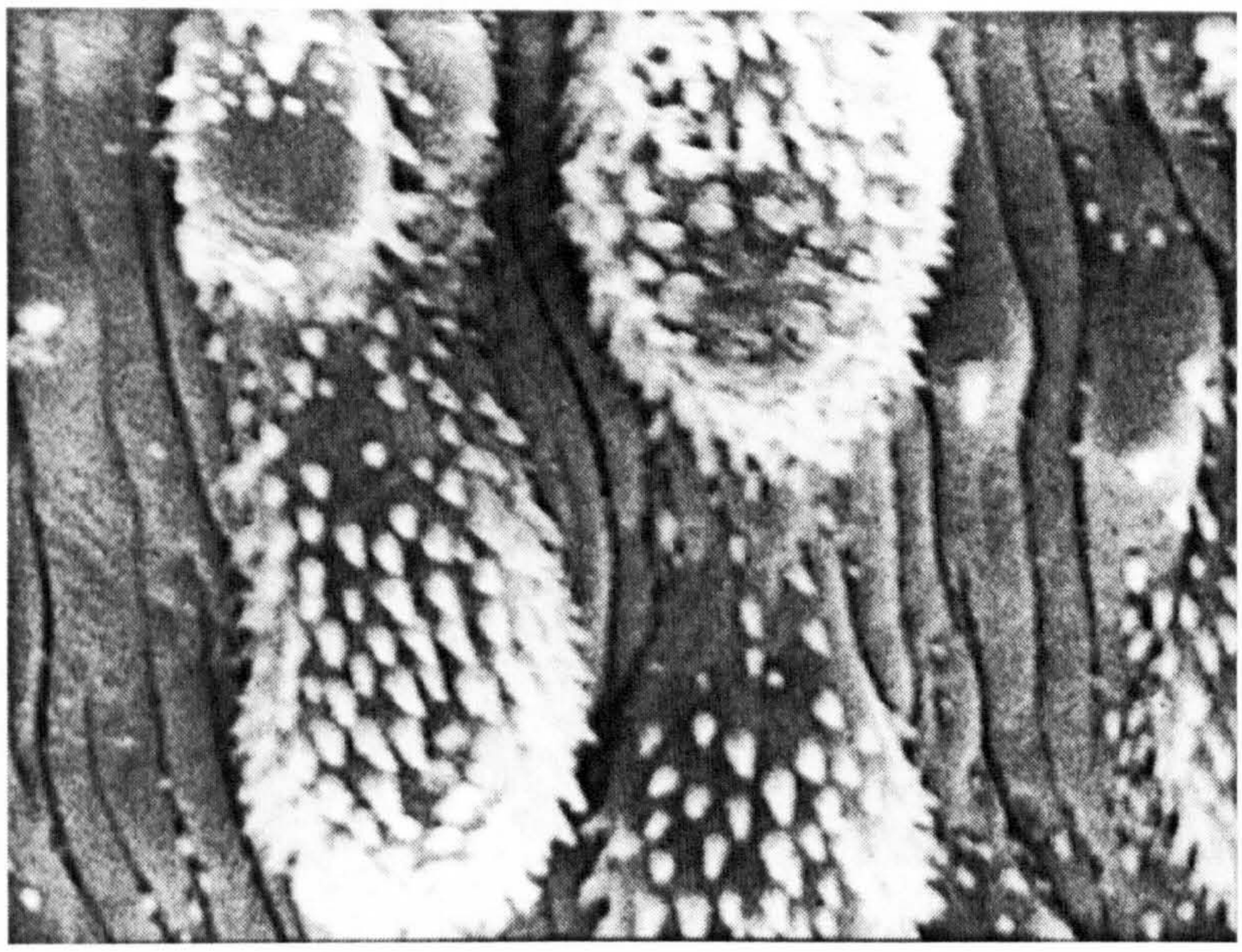
Figure 4.6 DMSO and DNNTZ effect on the tegumental surface of *S.mansoni*.



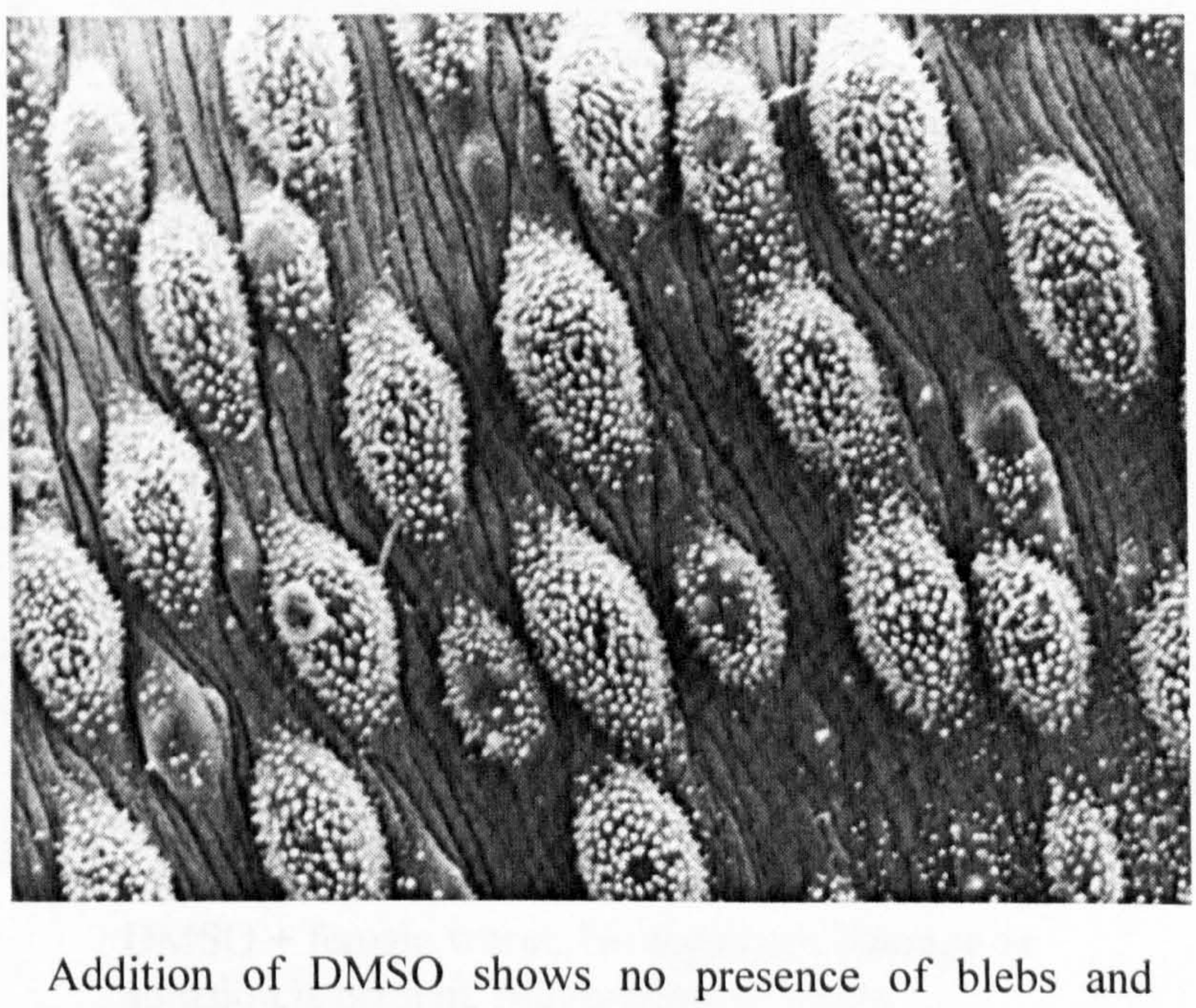
Addition of 10µg/ml DNNTZ does not cause paralysis or worm separation with *S. mansoni*. Magnification 45x. Similarly addition of DMSO (0.2%) does not result in worm pair separation.



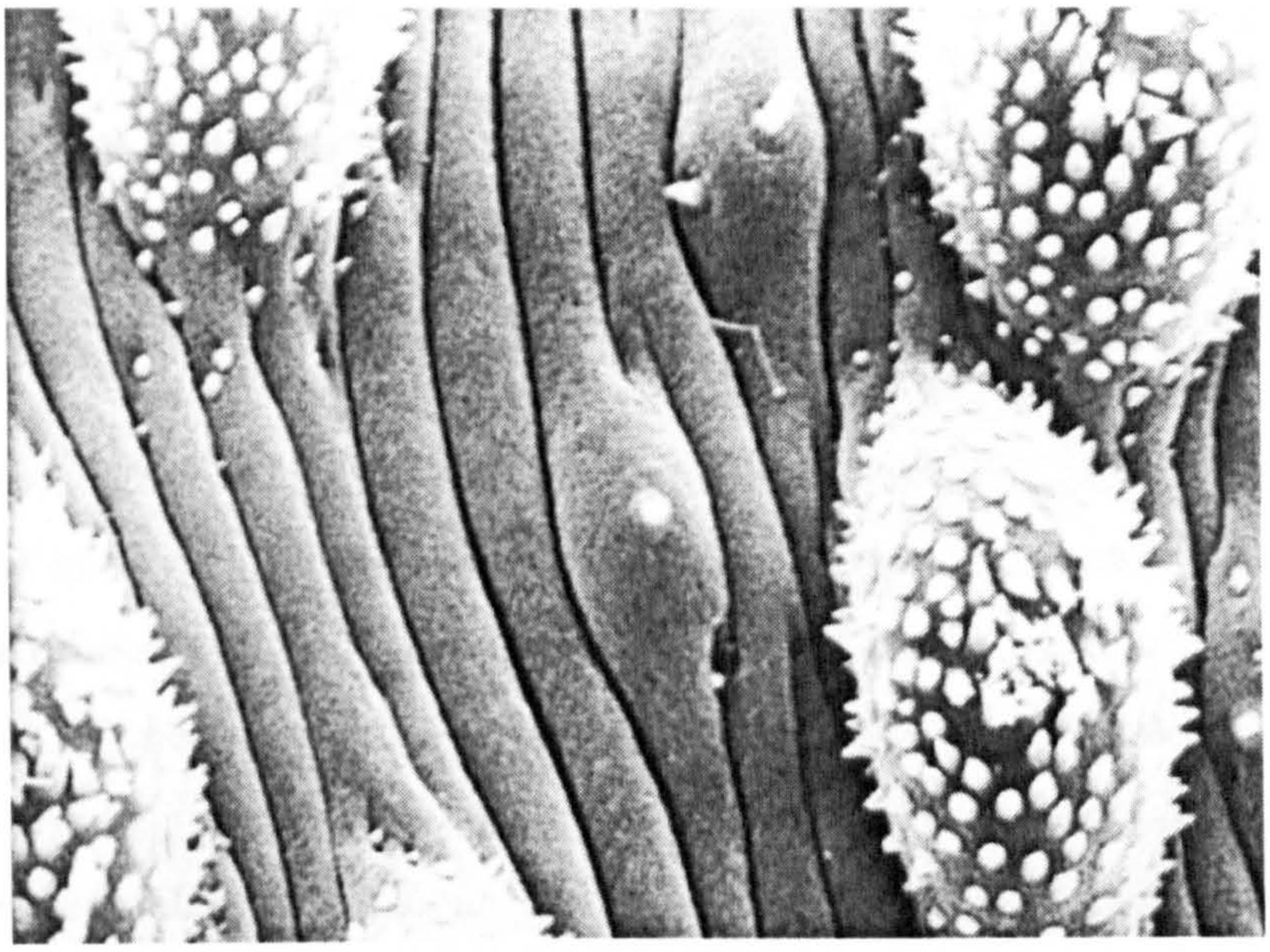
Addition of DNNTZ shows no presence of blebs and tegument of male schistosome appears intact. Magnification 1000x



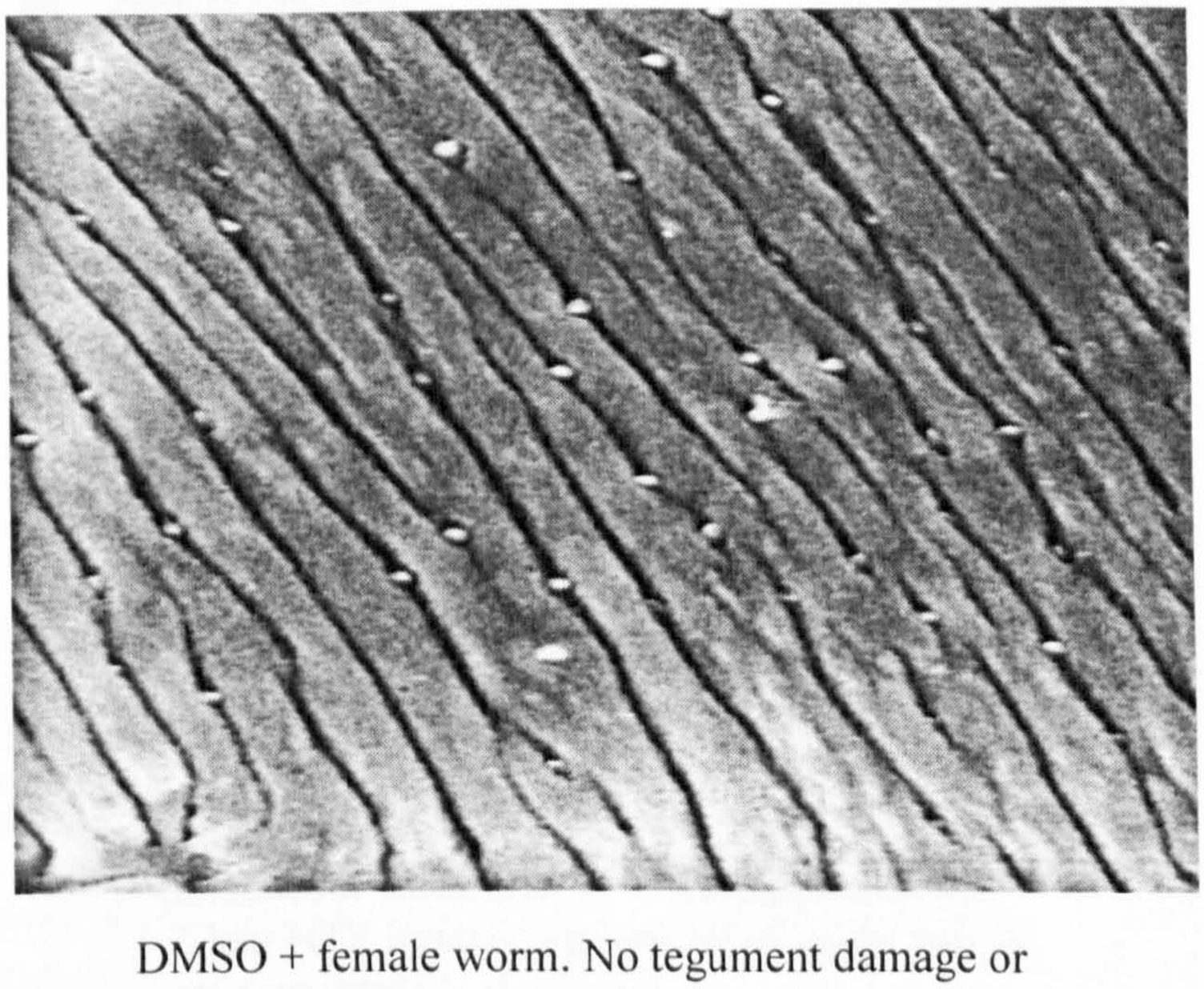
DNNTZ + male worm Magnification 3000x



Addition of DMSO shows no presence of blebs and tegument of male schistosome appears intact. Lack of effect is identical to that observed with DNNTZ.
Magnification 1000x



DMSO + male worm. No tegument damage is present. Magnification 3000x



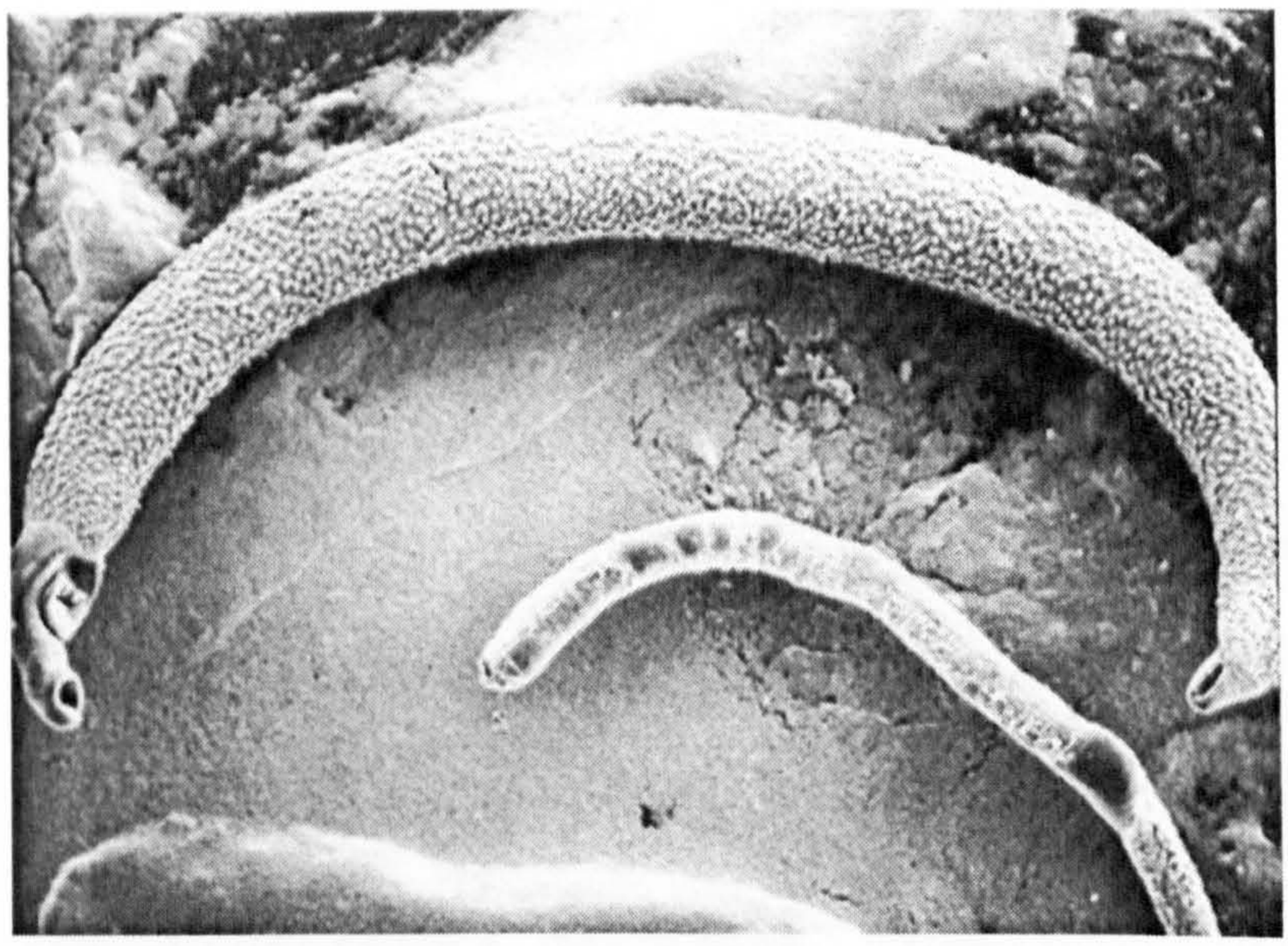
DMSO + female worm. No tegument damage or abrasion is present. Magnification 3000x

Fig 4.6: Adult worm pairs were exposed to 10μg/ml DNNTZ a) or the equivalent volume of DMSO b) for 1 hour. Examination of tegument was then performed using SEM. With the addition of DNNTZ no tegumental differences were observed compared to the DMSO control. Female tegument was also unaffected.

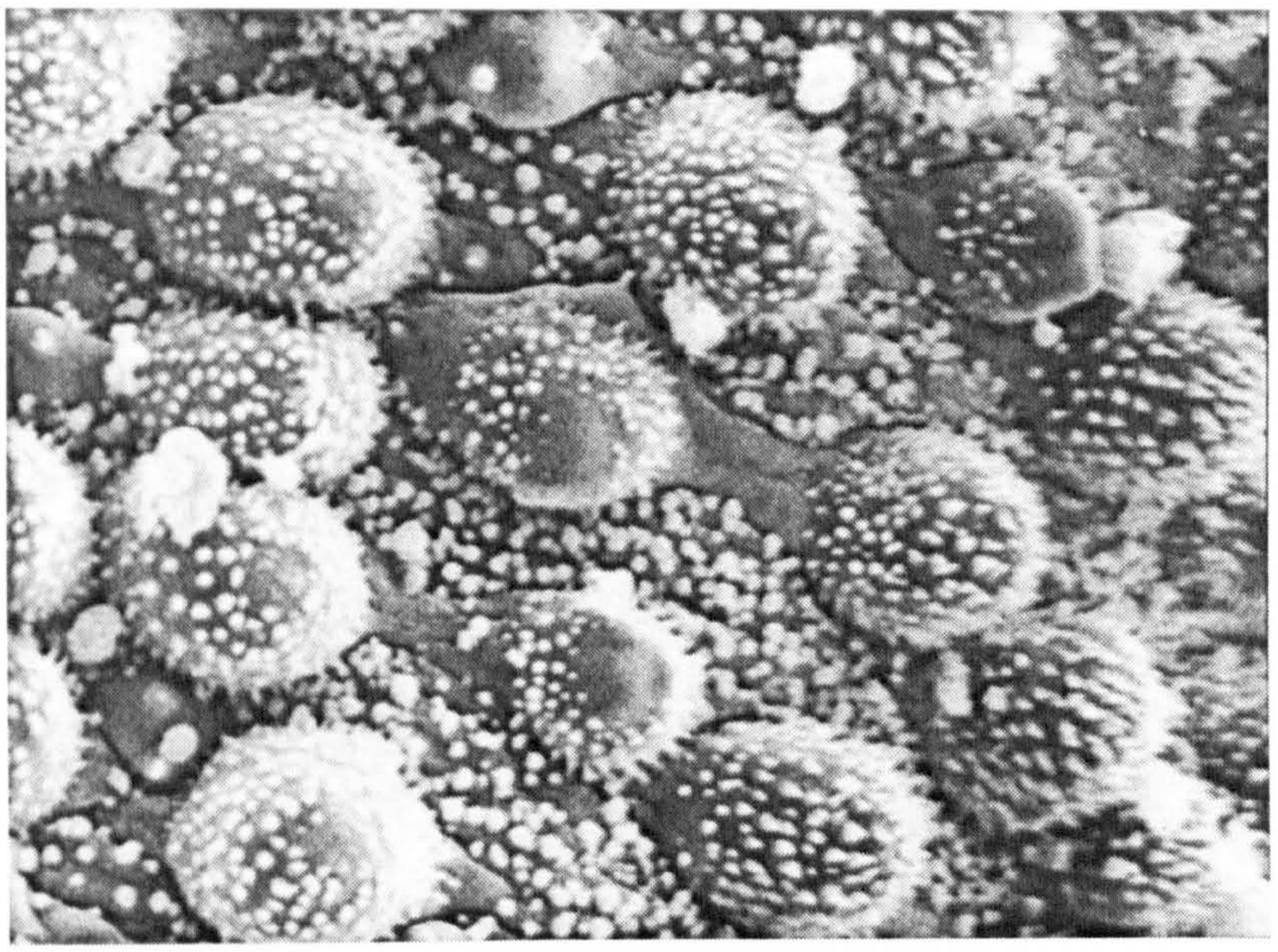
4.7.3 Effect of NTZ.

SEM examination of the samples showed that 1 hour exposure with 10µg/ml NTZ damaged the male and female schistosome tegument compared to that of the DMSO control. Males were more susceptible to the NTZ-induced damage than females. However, the extent of NTZ damage depended on the body part of the fluke. Damage to the male tegumental surface was extreme and characterised by blebbing due to the swelling of microvilli, and in some areas disruption of these structures had occurred forming lesions resulting in erosion and desquamation of the tegumental surface. The ventral as well as the dorsal surfaces of the male worm showed the same changes whereas the anterior part tended to be less damaged than the posterior part. With the female worm damage to the tegument was less clearly seen, but was characterised by an abraded appearance and showed swelling in some areas.

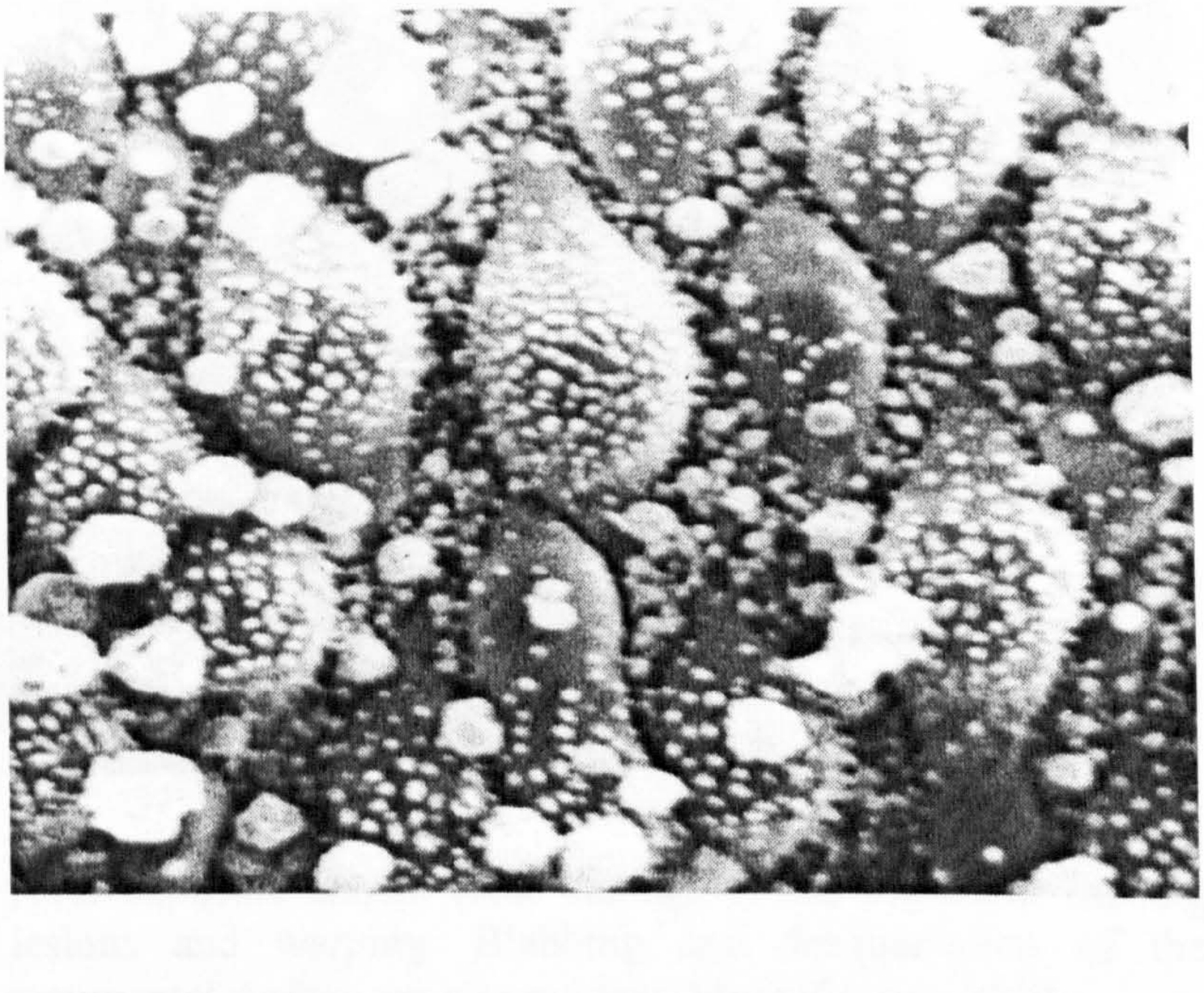
Figure 4.7 NTZ induced damage of *S.mansoni* tegumental surface.



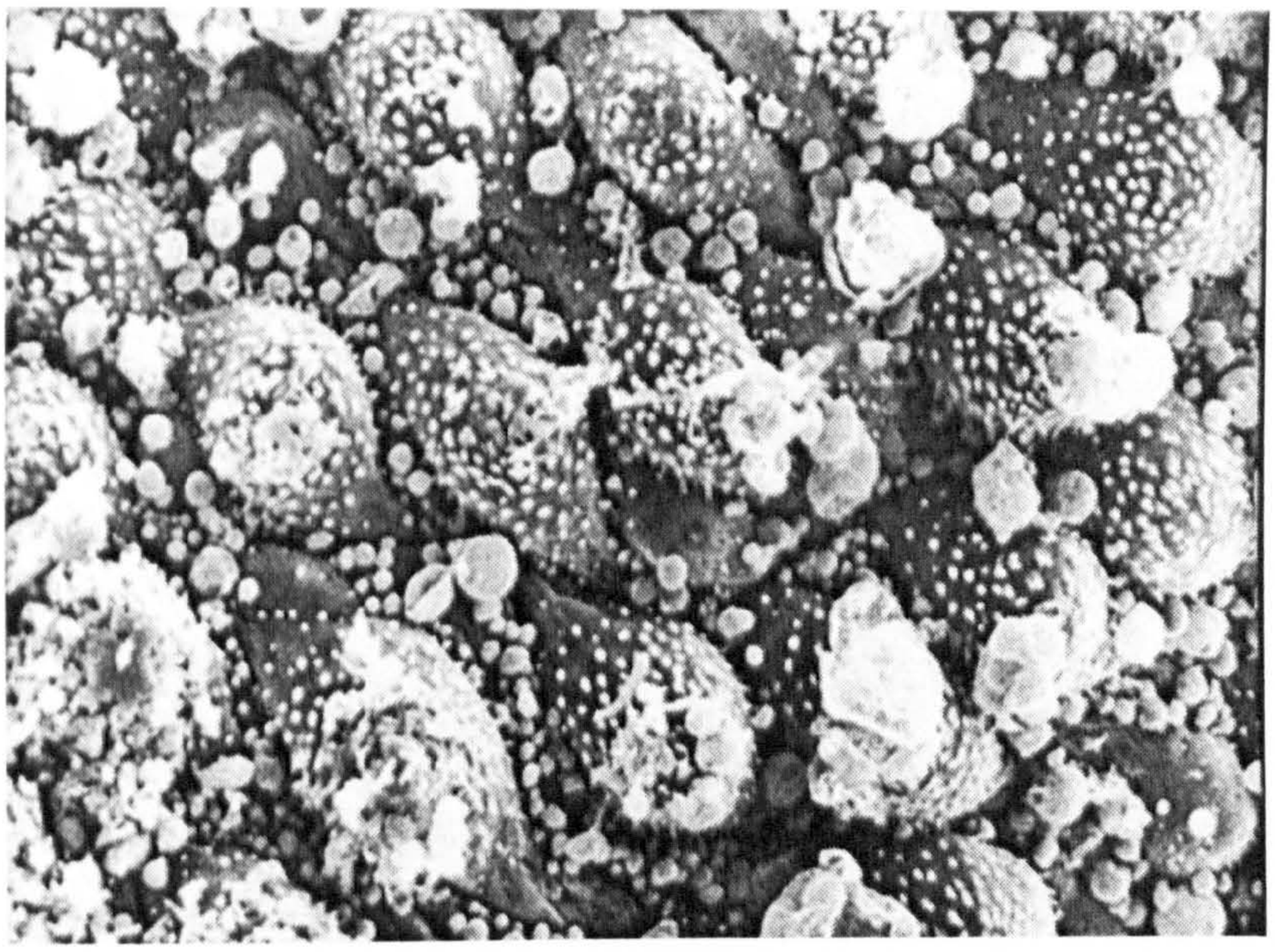
S.mansoni worm pair at 45x magnification. Clear NTZ induced separation of worm pair is evident. Male is above.



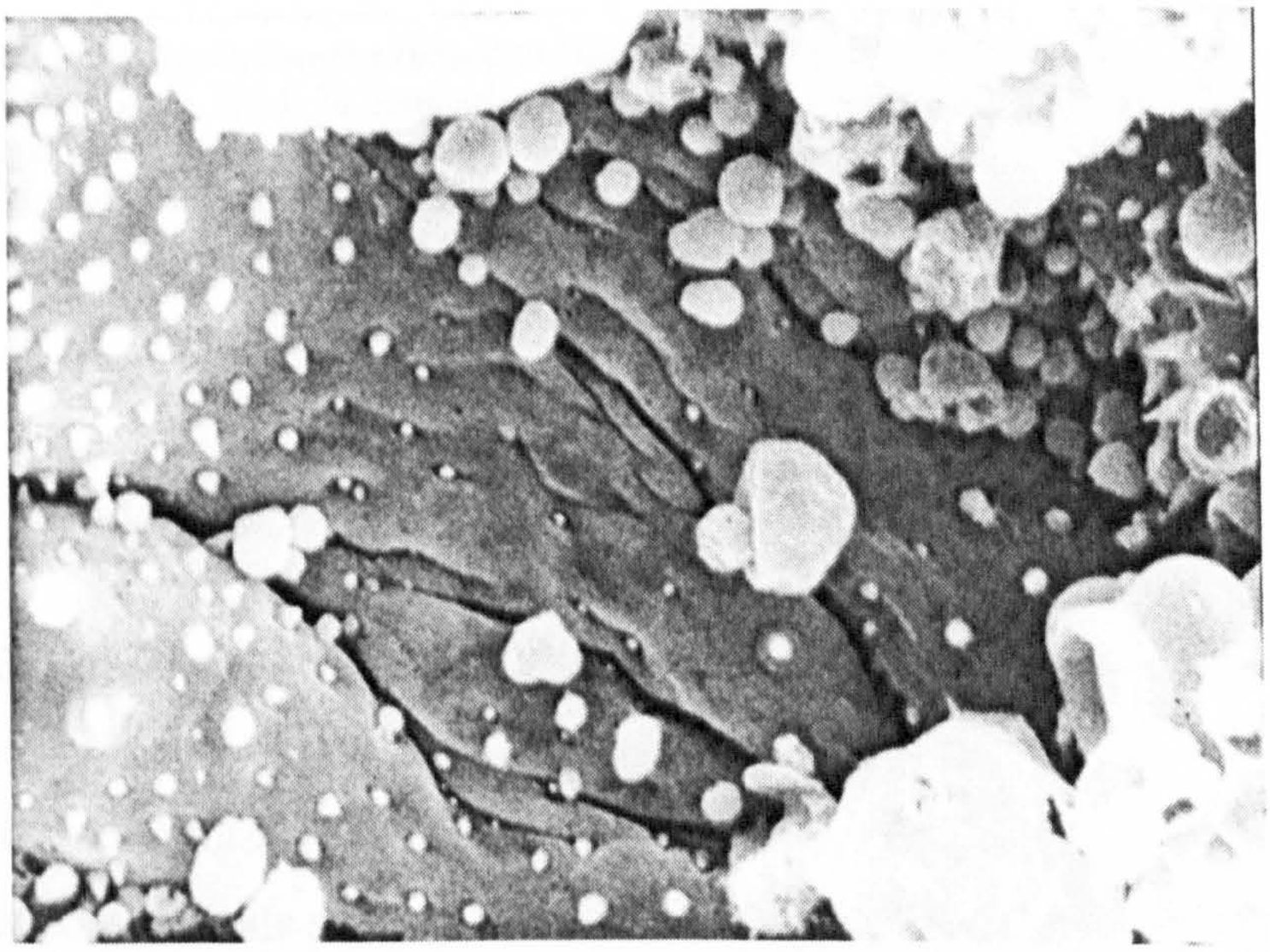
NTZ induced blebbing clearly evident on the tegumental surface of the male adult worm. Magnification 1500x



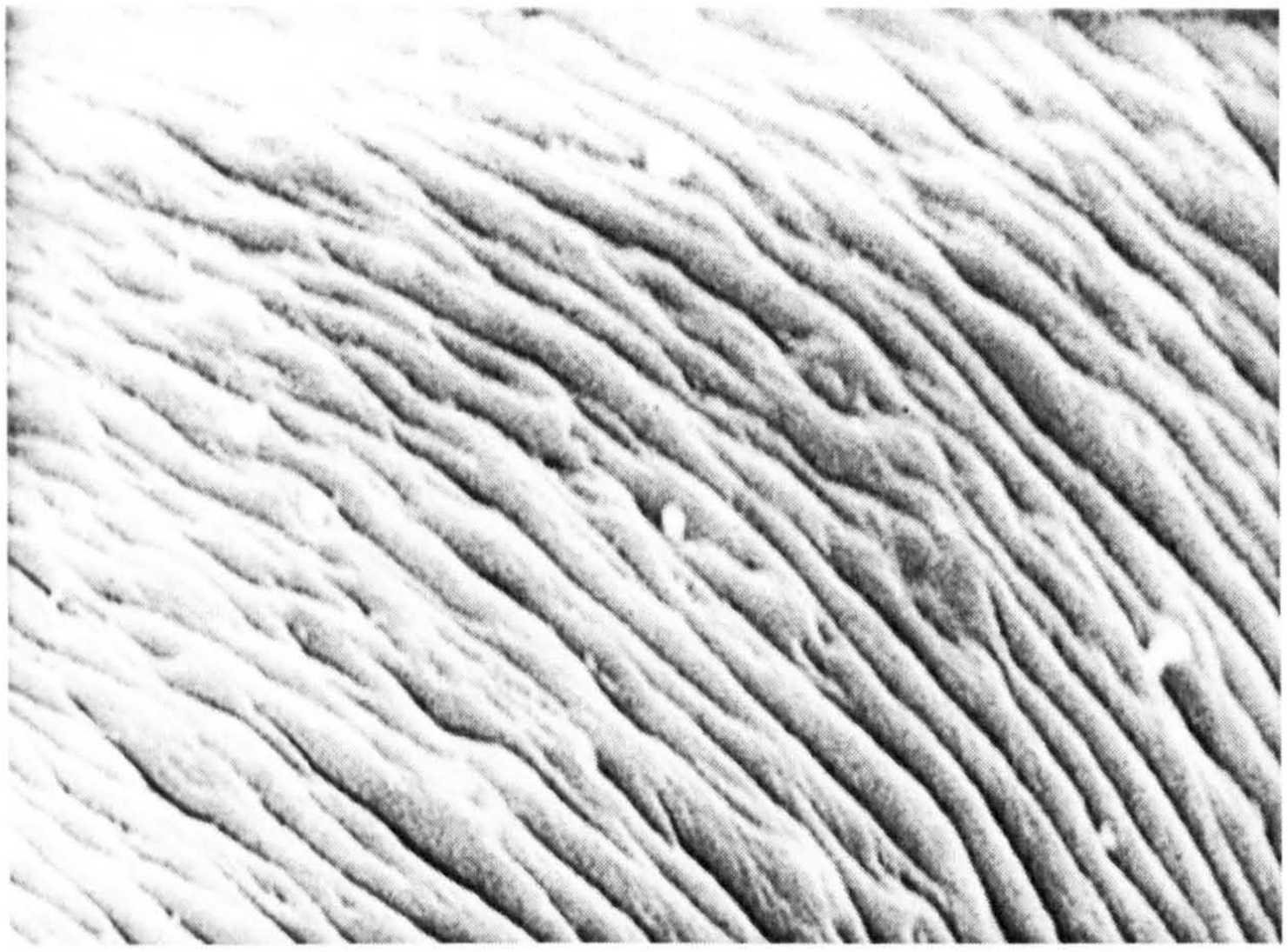
NTZ induced blebbing on tegumental surface of adult male worm. Magnification 1000x.



NTZ induced blebbing on tegumental surface of adult male worm. Magnification 1000x.



NTZ exposure shows clear damage to the tegument causing lesions and warping. Blebbing and desquamation of the tegumental surface are also evident. Magnification 3000x



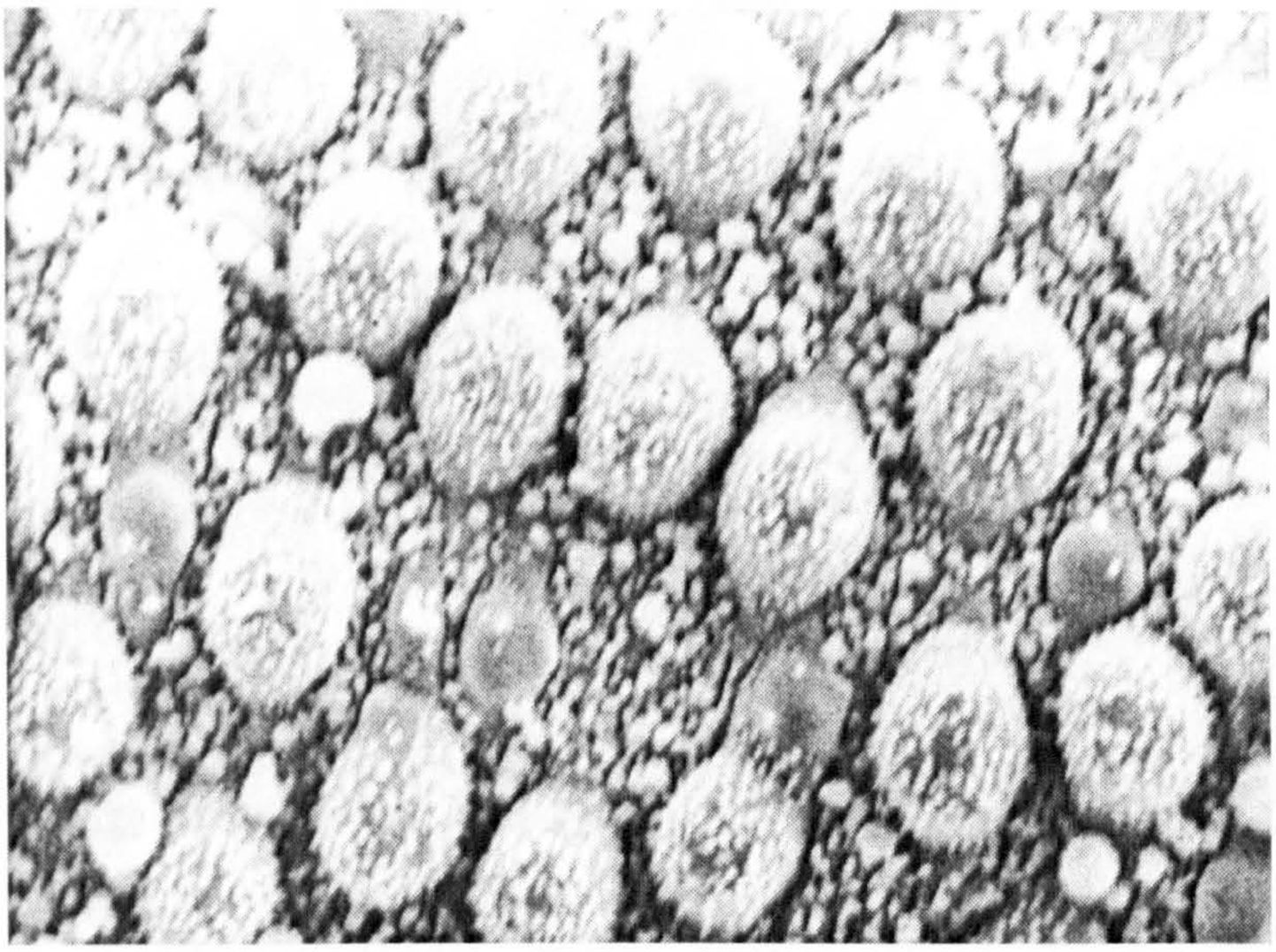
Addition of 10µg/ml causes abrasion and increased coarseness of female tegument. Effects are not as severe as those observed with male worms. Magnification 3000x

Fig 4.7: Adult worm pairs were exposed to 10µg/ml NTZ for 1 hour. Examination of tegument was then performed using SEM. In comparison to the DMSO control, addition of NTZ caused severe blebbing and desquamation of the male tegumental surface and caused the formation of lesions. Female tegument was significantly less affected but took on an abraded (rough) appearance and appeared more swollen compared to the DMSO control.

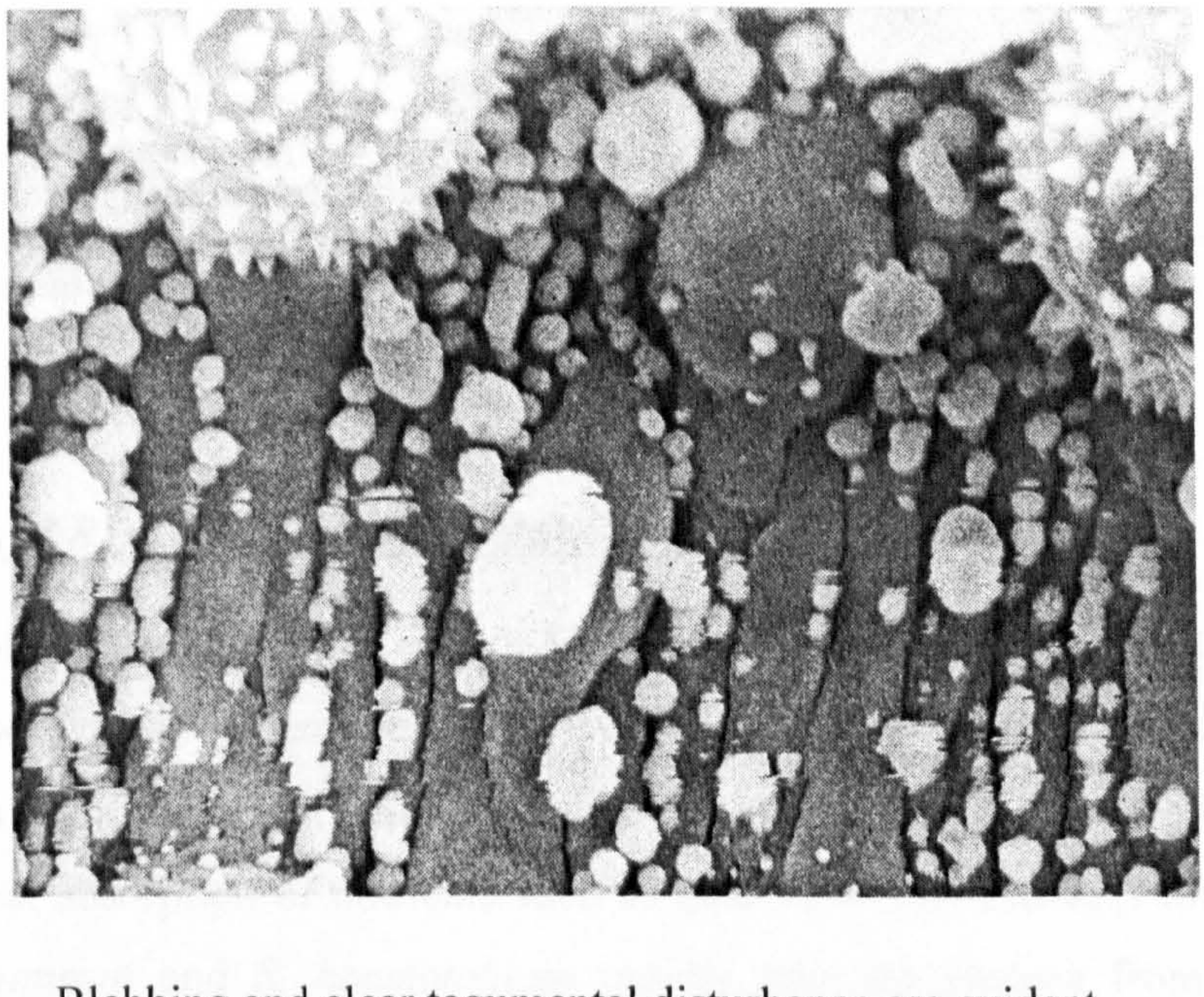
4.7.4 Effect of BZNT.

Effects of BZNT exposure on worms were similar to those observed with NTZ, and the compound caused blebbing and desquamation of the tegumental surface of the male. At higher magnifications, clear damage could be seen in the tegument characterised by the formation of lesions. Female tegument effect was identical to that observed with NTZ.

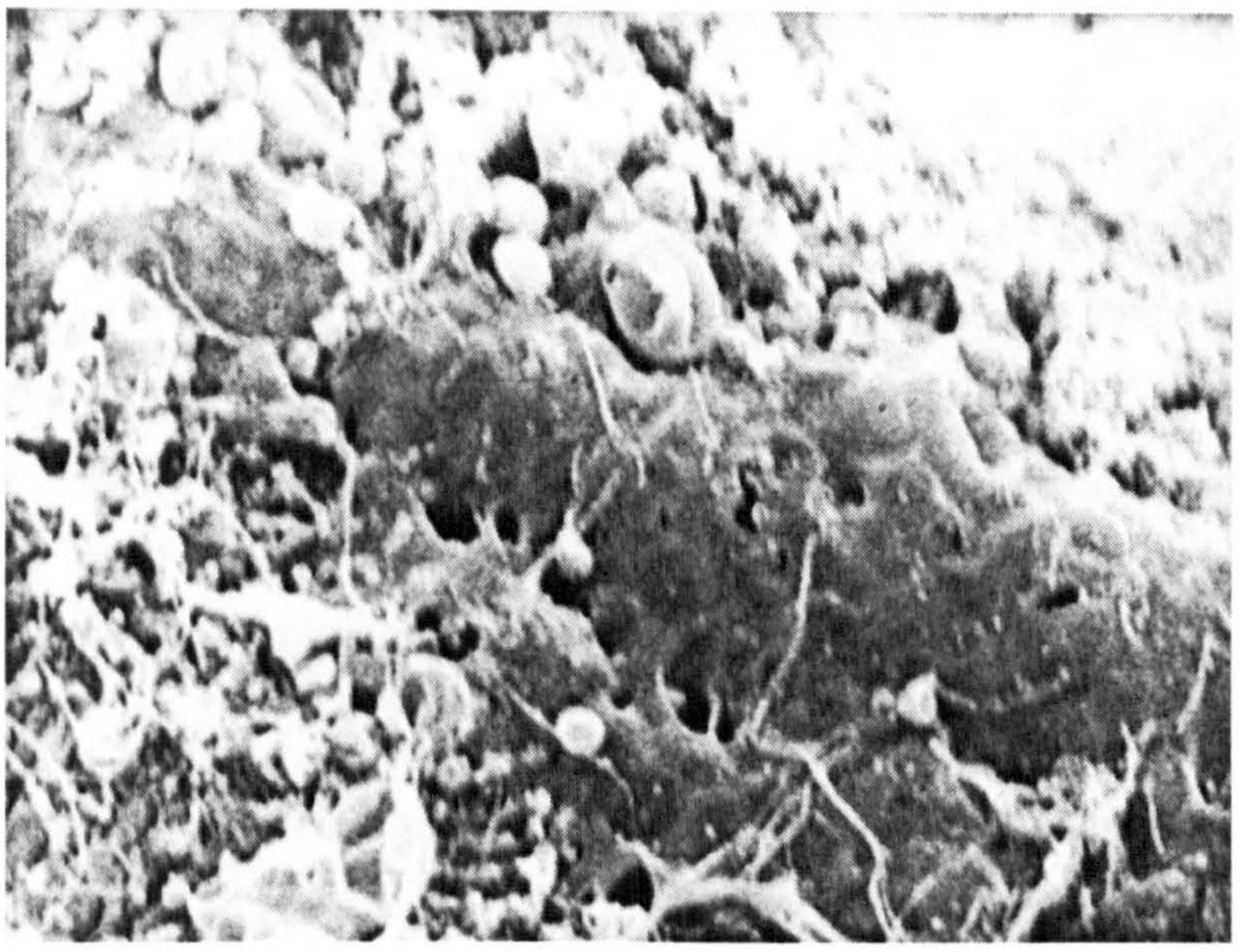
Figure 4.8 BZNT induced damage of *S.mansoni* tegumental surface.



BZNT induced blebbing on the tegumental surface of male *S. mansoni*. Magnification 1000x



Blebbing and clear tegumental disturbance are evident with BZNT. Magnification 3000x.



Addition of 10µg/ml BZNT causes severe damage to the tegument of male schistosomes. Damage is characterised by desquamation of tegumental surface, and the formation of lesions. BZNT induced damage appears more severe than that observed with NTZ.
Magnification x 3000

Fig 4.8: Adult worm pairs were exposed to 10µg/ml BZNT for 1 hour. Examination of tegument was then performed using SEM. In comparison to the DMSO control, addition of BZNT caused severe blebbing and desquamation of the male tegumental surface and caused the formation of lesions in a similar way to that observed with NTZ. Tegumental disruption however seemed more severe with BZNT than with NTZ even though drug induced effect was less marked. Female tegument like that observed with NTZ was significantly less affected.

4.8 Inhibition of *S. mansoni* glucose uptake by nitazoxanide.

The tegumental damage observed when *S. mansoni* was exposed to 10µg/ml NTZ is similar that reported with *Opisthorchis viverrini* (a liver fluke) when exposed to 10µg/ml praziquantel (Apinhasmit *et al*, 1988). Andrews & Thomas, 1979, have shown addition of PRAZ can affect absorption of nutrients such as glucose in *Hymenolepis diminuta*. Adult *Schistosoma mansoni* and *S. haematobium* rapidly take up glucose from the medium (Camacho and Agnew, 1995). In order to test whether NTZ/TIZ affected nutrient content, glucose uptake by *S. mansoni* schistosomula and adults was examined in the presence of NTZ and other drugs in medium 169 without FCS.

4.8.1 Effect of NTZ on glucose uptake by schistosomula.

Pre-incubation for 30 minutes with 20μg/ml PRAZ, NTZ, TIZ, DNNTZ and BZNT inhibited glucose uptake by *S. mansoni* schistosomula (table 4.5). NTZ and TIZ gave the highest inhibition, with both demonstrating a 60% drop in glucose uptake compared to the DMSO control and both showed highly significant differences. DNNTZ inhibited glucose uptake by 19% at 20μg/ml, even though it had no effect on worm motility or morphology at this concentration. At lower concentrations the praziquantel inhibition of glucose uptake changed little from 44% at 20μg/ml to 58% at both 10μg/ml and 1μg/ml. NIC showed no inhibition of glucose uptake at all concentrations used, however NIC was seen to increase uptake suggesting an effect on electron transport (uncoupling) or glycolysis.

Though both NTZ and PRAZ inhibited glucose uptake at 10 and 20μg/ml, the killing effect *in vitro* of NTZ and TIZ was more rapid than that observed with PRAZ. This suggests that though glucose uptake in schistosomula was affected with addition of both drugs, the more rapid death observed with NTZ shows that drug induced inhibition of nutrient absorption is not the primary mechanism of action of NTZ/TIZ.

Table 4.5 Effect of NTZ/TIZ on glucose uptake in *S.mansoni* schistosomula.

DRUG (µg/ml)	Deoxy-glucose uptake ± s.d ng glucose/ µg protein/ min. (n=3)	% Inhibition	P value N.S = not significant
DMSO (0.2%)	7.8 ± 0.4	0	control
PRAZ (20)	4.4 ± 0.6	44	P < 0.0001
PRAZ (10)	3.3 ± 0.2	58	P < 0.000001
PRAZ (1)	3.3 ± 0.2	58	P < 0.000001
NTZ (20)	3.1 ± 0.5	60	P < 0.00001
NTZ (10)	4.2 ± 0.2	46	P < 0.00001
NTZ (1)	7.8 ± 0.2	0	N.S
TIZ (20)	3.1 ± 0.5	60	P < 0.00001
TIZ (10)	6.0 ± 0.4	23	P < 0.001
TIZ (1)	8.4 ± 0.6	- 8	N.S
NIC (20)	8.2 ± 0.2	- 5	N.S
NIC (10)	8.7 ± 0.6	- 12	N.S
NIC (1)	8.2 ± 0.2	- 5	N.S
DNNTZ (20)	6.3 ± 0.4	19	P < 0.01
DNNTZ (10)	7.0 ± 0.3	10	N.S
DNNTZ (1)	8.2 ± 0.2	- 5	N.S
BZNT (20)	6.6 ± 0.4	15	P < 0.01
BZNT (10)	6.9 ± 0.3	12	P < 0.02
BZNT (1)	8.5 ± 0.2	- 9	N.S

TABLE 4.5: The above table shows a typical result of glucose uptake studies. Praziquantel at all 3 concentrations inhibited uptake of glucose in schistosomula, consistent with the earlier findings of Harder, 1987. NTZ and TIZ, also significantly inhibited glucose uptake at 20µg/ml and 10µg/ml but not at 1µg/ml. At 10µg/ml the inhibition of NTZ is greater than that of TIZ. DNNTZ showed a significant inhibition of glucose uptake only at the highest dose even though no effect on worm motility was observed. NIC, though having very strong paralysing and toxic effect, at these concentrations did not significantly affect glucose uptake. However uptake was stimulated suggesting a possible effect on glycolysis. For concentration conversions from µg/ml to µM see appendix IV.

Figure 4.9 <u>Percentage of glucose uptake in drug exposed schistosomula.</u>

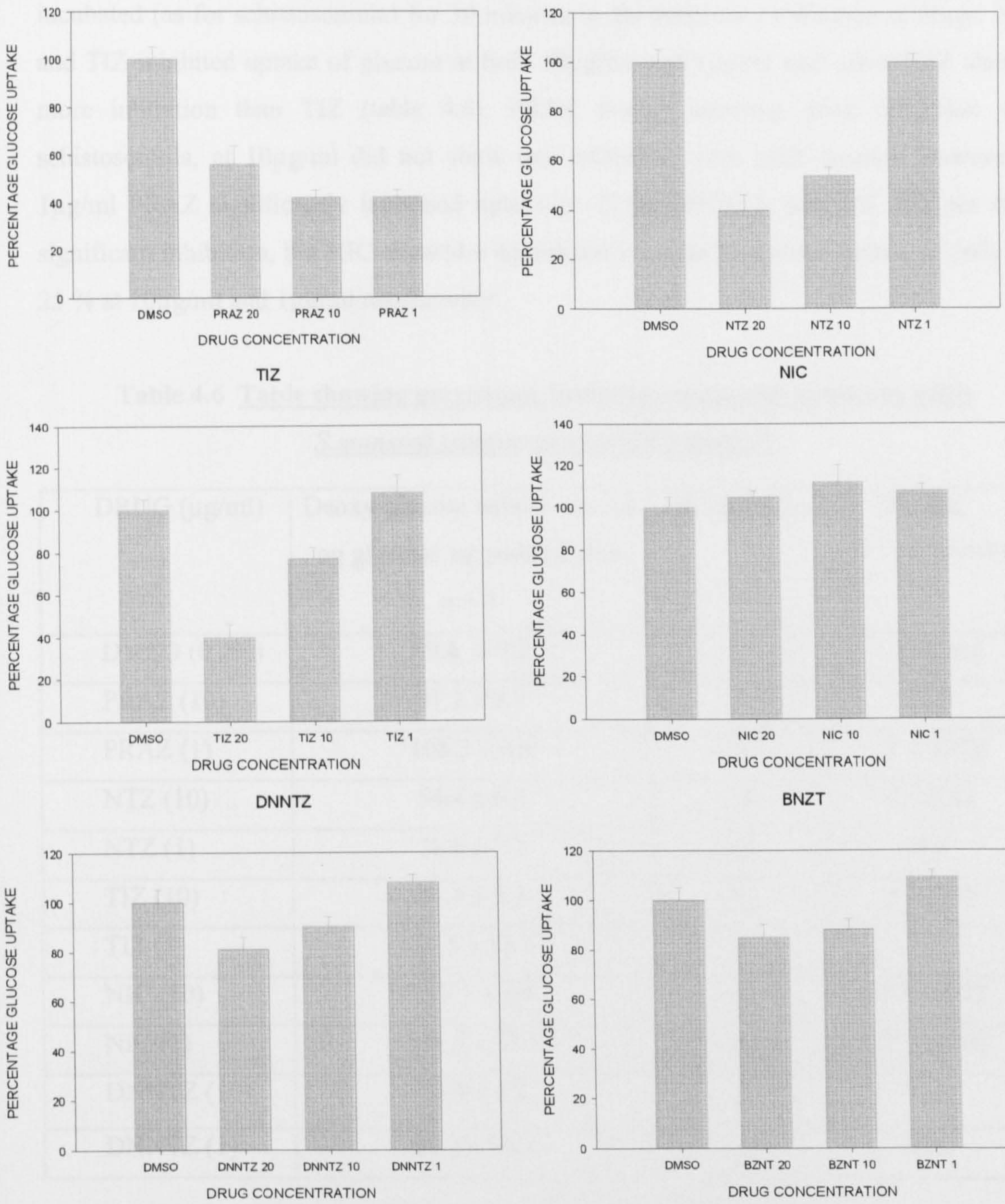


FIG 4.9: Drugs at concentrations of 20, 10 or 1µg/ml were added to wells containing 50 schistosomula in 100µl medium 169 and incubated for 30 minutes. [^{3}H] deoxy-glucose was added, schistosomula were washed and glucose uptake measured in a ß-counter. Addition of PRAZ, NTZ or TIZ showed a clear inhibitory effect on glucose uptake. DNNTZ and BZNT showed a slight effect at higher concentrations. NIC did not affect glucose uptake, but increased it suggesting an effect on glycolysis.

4.8.2 Effect of NTZ on glucose uptake by Adult *S. mansoni.*

Glucose uptake was measured in 100µl samples containing 2 pairs of adult *S. mansoni* pre-incubated (as for schistosomula) for 30 minutes in the presence or absence of drugs. NTZ and TIZ inhibited uptake of glucose at both 10µg/ml and 1µg/ml and again NTZ showed more inhibition than TIZ (table 4.6). PRAZ though showing good inhibition with schistosomula, at 10µg/ml did not show any inhibition with adult worms. However at 1µg/ml PRAZ significantly increased uptake (+ 21%). DNNTZ, and NIC did not show significant inhibition, but NIC showed a significant increase in glucose uptake of 26% and 25 % at 10µg/ml and 1µg/ml respectively.

Table 4.6 Table showing percentage inhibition of glucose uptake in adult *S.mansoni* compared to DMSO control.

DRUG (µg/ml)	Deoxy-glucose uptake ± s.d ng glucose/ µg protein/ min. n = 3	% Inhibition	P value N.S = not significant
DMSO (0.2%)	89.6 ± 9.2	0	Control
PRAZ (10)	91.3 ± 2.7	- 1	N.S
PRAZ (1)	108.3 ± 6.6	- 21	P < 0.002
NTZ (10)	54.4 ± 6.3	39	P < 0.01
NTZ (1)	78.6 ± 7.1	12	N.S
TIZ (10)	61.7 ± 9.3	31	P < 0.03
TIZ (1)	79.5 ± 15.9	11	N.S
NIC (10)	113.1 ± 8.8	- 26	P < 0.002
NIC (1)	111.7 ± 17.1	- 25	P < 0.002
DNNTZ (10)	86.9 ± 3.2	3	N.S
DNNTZ (1)	98.2 ± 11.4	- 10	N.S

TABLE 4.6: The above table shows a typical result of glucose uptake studies with adults. NTZ and TIZ, significantly inhibited glucose uptake at 10µg/ml but not at 1µg/ml. PRAZ at 1µg/ml and NIC at both concentrations stimulated uptake of glucose in schistosomes, with increased uptake being significantly different to that of the DMSO control. The slight inhibition of glucose uptake by DNNTZ at 10µg/ml nor increased uptake at 1µg/ml were not significantly different to the DMSO control. For concentration conversions from µg/ml to µM see appendix IV

Figure 4.10: Percentage of glucose uptake in drug exposed *S.mansoni* adults.

DMSO represents 100% uptake

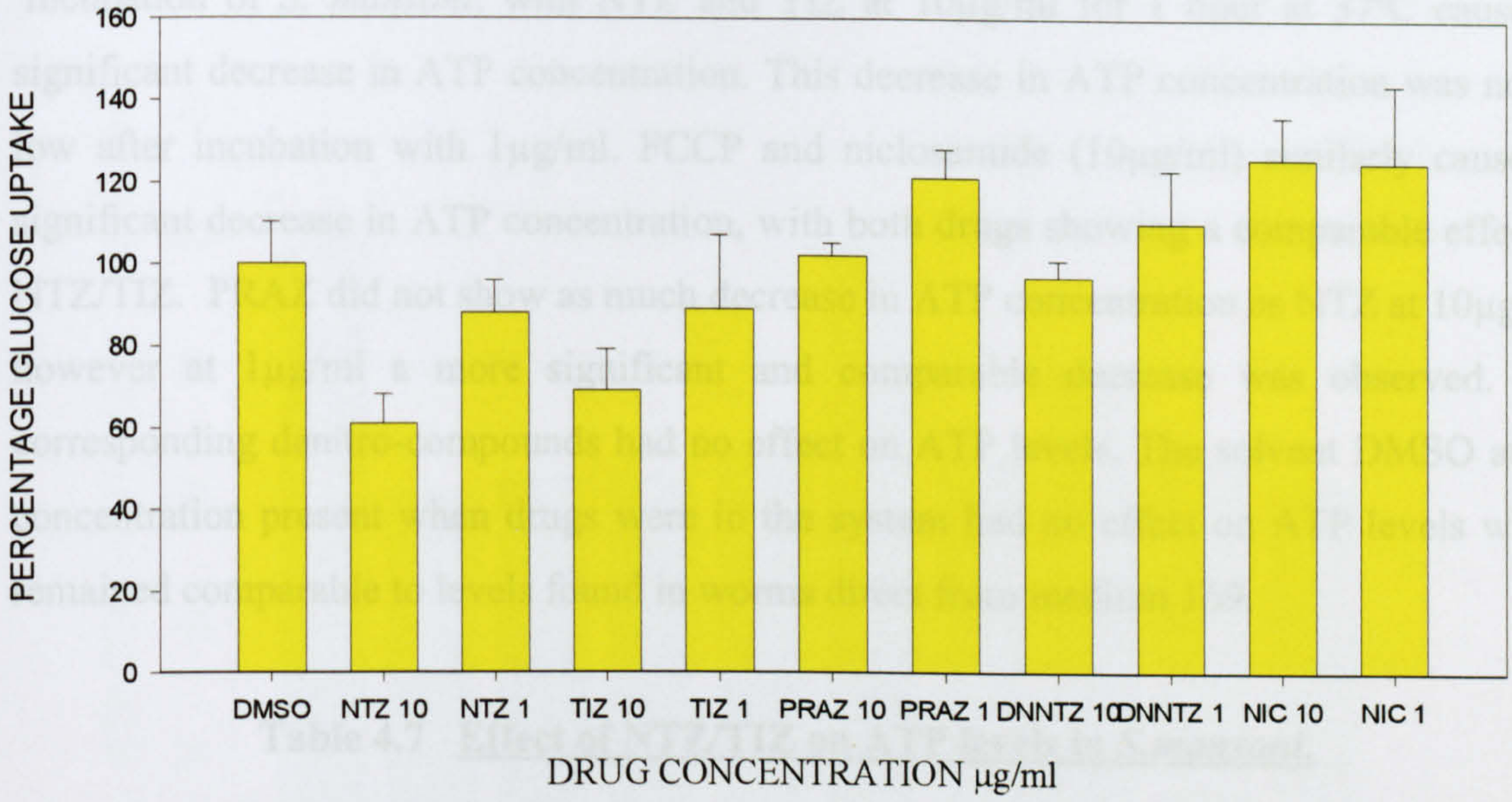


FIG 4.10: Drugs at concentrations of 10 and 1µg/ml were added to wells containing 2 pairs of adult *S.mansoni* in 100µl medium 169. After 30 minutes drug exposure followed by 20 minute addition of [^{3}H] deoxy-glucose, worms were washed and glucose uptake measured in a ß-counter. Addition of NTZ or TIZ showed a clear effect on glucose uptake. DNNTZ showed a very small inhibition at the higher concentrations. NIC and PRAZ did not affect glucose uptake but appeared to stimulate it.

The results show that NTZ and TIZ are significantly inhibiting glucose uptake in schistosomula and adults at higher concentrations than 1µg/ml. This may be due to the damaging effect that these drugs have on the tegument of the worm. However owing to the rapid paralysis occurring in adults with addition of 1µg/ml NTZ/TIZ and no evident inhibition of glucose at this concentration, it can be assumed that impairment of nutrient absorption is independent of NTZ/TIZ observed paralysis.

4.9 Examination of effect of NTZ on ATP levels in *S.mansoni.*

Luminometric assays were carried out with adult *S. mansoni* in the same way as for *C. elegans* described in section 3.11. After 30 minutes drug exposure in the absence of serum,

worms were dissolved in 0.1 M NaOH/ EDTA and plates read for luminescence in a Wallac ß-counter.

Incubation of *S. mansoni*, with NTZ and TIZ at 10µg/ml for 1 hour at 37°C caused a significant decrease in ATP concentration. This decrease in ATP concentration was not as low after incubation with 1µg/ml. FCCP and niclosamide (10µg/ml) similarly caused a significant decrease in ATP concentration, with both drugs showing a comparable effect to NTZ/TIZ. PRAZ did not show as much decrease in ATP concentration as NTZ at 10µg/ml, however at 1µg/ml a more significant and comparable decrease was observed. The corresponding denitro-compounds had no effect on ATP levels. The solvent DMSO at the concentration present when drugs were in the system had no effect on ATP levels which remained comparable to levels found in worms direct from medium 169.

Table 4.7 Effect of NTZ/TIZ on ATP levels in *S.mansoni.*

Treatment	DMSO (A) 0.2 %	NTZ (B) 10µg/ml	NTZ (C) 1µg/ml	TIZ (D) 10µg/ml
Mean [ATP] nmol mg protein^{-1}.	16.3	9.9	10.3	10.3
S.D.	3.5	3.3	2.7	3.1
Treatment	TIZ (E) 1µg/ml	DNTIZ (F) 10µg/ml	NIC (G) 10µg/ml	PRAZ (H) 10µg/ml
Mean [ATP] nmol mg protein^{-1}.	10.9	16.1	10.7	13.2
S.D.	2.4	3.3	3.0	2.7
Treatment	PRAZ (I) 1µg/ml	FCCP (J) 10µg/ml		
Mean [ATP] nmol mg protein^{-1}.	10.9	10.3		
S.D.	2.9	3.6		

Table 4.7: Mean [ATP] was calculated for each drug and compared to DMSO control. The above table shows the typical values of the drug effects on ATP levels in nmol mg protein^{-1}. The data for several experiments were hence incorporated and analysed in figure 4.11.

Figure 4.11 Effect of NTZ/TIZ on ATP content of *S. mansoni*.

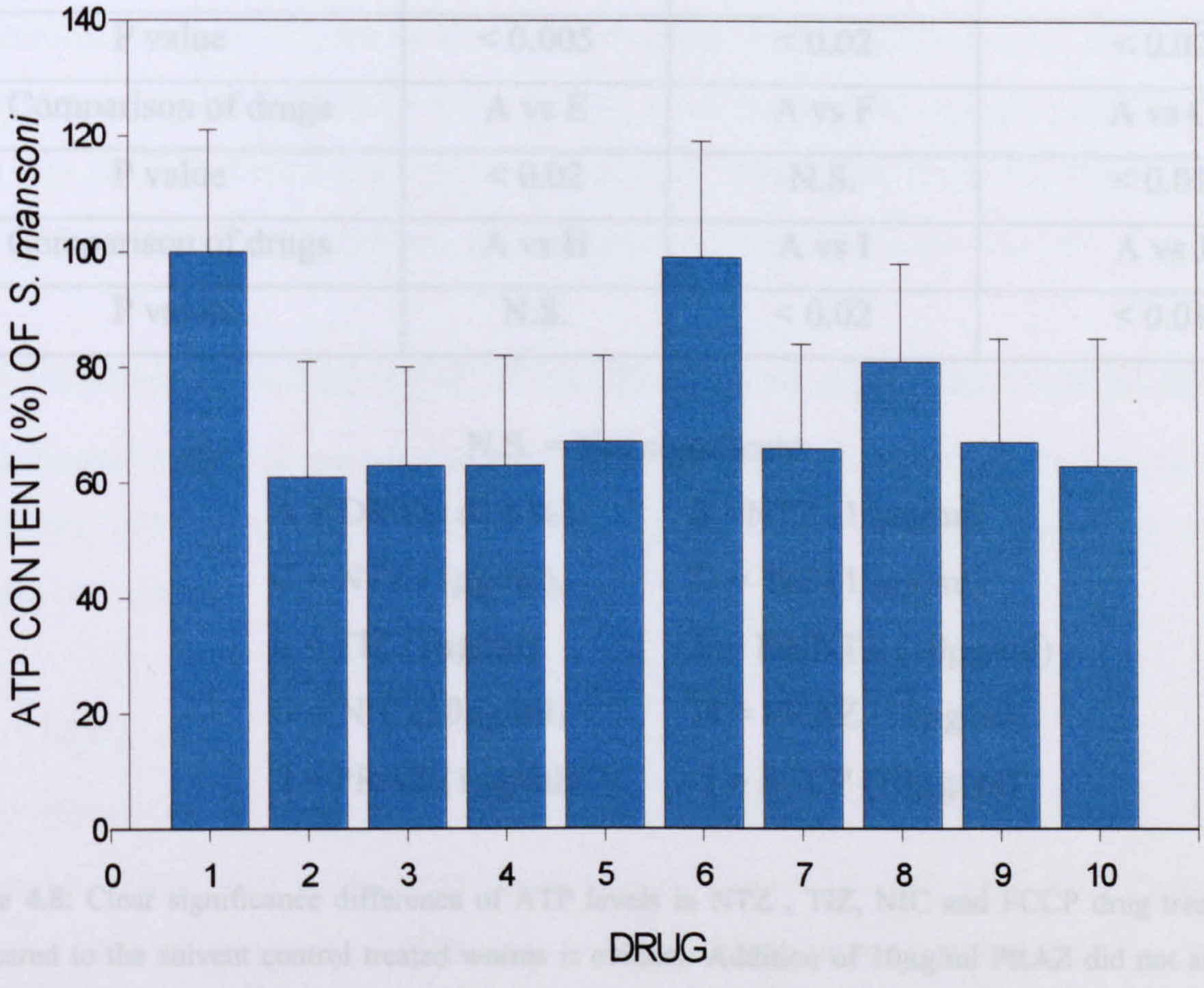


Fig 4.11: *S. mansoni* were incubated for 30 minutes at 37°C with NTZ (10 or 1µg/ml), TIZ (10 or 1µg/ml), DNNTZ (10µg/ml), NIC (10µg/ml), PRAZ (10 or 1µg/ml), FCCP (10µg/ml) or DMSO (0.2%). After solubilising in alkali, ATP was determined as described in the methods. All assays were carried out in triplicate and similar results were obtained in separate experiments. Significant differences between values were calculated using a Student t-test (see table 4.8).

Table 4.8 Significance testing of ATP levels in drug exposed *C.elegans*.

Comparison of drugs	A vs B	A vs C	A vs D
P value	< 0.005	< 0.02	< 0.02
Comparison of drugs	A vs E	A vs F	A vs G
P value	< 0.02	N.S.	< 0.01
Comparison of drugs	A vs H	A vs I	A vs J
P value	N.S.	< 0.02	< 0.01

N.S. = Not significant.

A = DMSO (0.2 %), B =NTZ (10μg/ml)

C = NTZ (1μg/ml), D = TIZ (10μg/ml)

E = TIZ (1μg/ml) F = DNNTZ (10μg/ml)

G = NIC (10μg/ml) H = PRAZ (10μg/ml)

I = PRAZ (1μg/ml) J = FCCP (10μg/ml)

Table 4.8: Clear significance difference of ATP levels in NTZ , TIZ, NIC and FCCP drug treated worms compared to the solvent control treated worms is evident. Addition of 10μg/ml PRAZ did not significantly reduce ATP levels, whereas 1μg/ml did. DNNTZ did not significantly reduce ATP levels in *S. mansoni*.

4.10 Assay of nitroreductase activity in *S.mansoni* with NTZ and TIZ.

Niridazole, an antischistosomal nitrothiazole derivative, is reduced by adult *Schistosoma mansoni* to one or more reactive intermediates (Tracy et al, 1983). Studies were therefore carried out to examine whether reduction of NTZ/TIZ occurred in the presence of *S, mansoni*.

Anaerobic conditions were created by purging a septum-sealed cuvette with argon in the presence of an oxygen-scavenging enzymatic system. On the mixing of the *S. mansoni* homogenate (0.3mg/ml protein) with NADH (or NADPH) and glucose-6-phosphate, however, the absorbance of NTZ/TIZ at 412nm was unchanged. Using niridazole instead to

test nitroreductase activity, also gave a negative result in contrast to the findings of Tracy *et al*, (1983) where addition of *S. mansoni* homogenate caused disappearance of absorbance of niridazole at 400nm. This may be due to the differences in the maintaining of anaerobic conditions. Tracy *et al*, used an anaerobic chamber, with a side-arm attached to the cuvette to ensure anaerobic conditions were present at all times. The septum-sealed cuvette used here may not have been completely satisfactory. It is therefore likely that some oxygen may still have remained in the system, and this could have inhibited the enzyme.

4.11 Examination of NTZ action on egg hatching, miracidia and cercariae motility.

Praziquantel affects *S. mansoni* egg hatching and the motility of the free-living miracidia (Andrews, 1978). For comparison, NTZ and TIZ and other Romark compounds were tested for similar effects.

4.11.1 Eggs.

Eggs placed under light hatch very rapidly, releasing the fast swimming, free-living miracidia. When eggs were pre-exposed (for 30 minutes) to 10µg/ml and 1µg/ml NTZ or TIZ and then illuminated, no inhibition of egg hatching was observed, and the majority of eggs hatched within 30 minutes. When eggs were exposed to 10µg/ml and 1µg/ml PRAZ, egg-hatching was clearly inhibited (as reported by Andrews, 1978) with many eggs remaining un-hatched after 2 hours. DNNTZ and DMSO (0.2%) had no inhibitory effect on egg-hatching.

4.11.2 Miracidia.

No initial effect on miracidial swimming speed or morphology was observed with 10µg/ml and 1µg/ml NTZ or TIZ. However, approximately half an hour after drug exposure, miracidia exposed to either drug became sluggish moving less rapidly than the DMSO control. After 1 hour exposure to NTZ and TIZ all miracidia became immobile, and many were swollen and rounded. No recovery of the miracidia was observed. PRAZ at 10µg/ml and 1µg/ml also slowed down miracidial movement. The effects however, were not as strong as those of NTZ, since after 1 hour in PRAZ, the miracidia were still able to swim.

DNNTZ and the solvent control DMSO (0.2%) had no effect on miracidial movement or morphology.

4.11.3 Cercariae.

Studies were also carried out on cercariae to see if Romark compounds had any effect on motility or viability. On exposure to 10μg/ml and 1μg/ml NTZ and TIZ, cercariae showed sluggishness after 30 minutes and were unable to move properly. After 1 hour cercariae were unable to swim, although the tail was able to move normally. Cercariae also showed some slight morphological change with NTZ and TIZ, characterised by a crookedness in the tail and swelling of the head. PRAZ and NIC at 1μg/ml also slowed down cercarial swimming and had a similar effect on motility to that of NTZ and TIZ but did not induce any morphological changes. DNNTZ and DMSO had no effect on motility or morphology of cercariae.

From these results, NTZ and TIZ show clear activity on all stages of the schistosome life-cycle except the egg. DNNTZ and DMSO showed no effect on any of the life cycle stages.

4.12 Examination of molluscicidal properties of NTZ.

The molluscicidal activity of BZNT 2- benzamido-5-nitrothiazole and NIC niclosamide have been recently studied (Dreyfuss, 1996: Rondelaud, 1996). Potential NTZ and TIZ activity as molluscicides was examined at 0.5mg/litre and 1mg/litre using niclosamide as a positive control and DMSO as a negative control. The snails used were *Biomphalaria glabrata*, the intermediate host of *S. mansoni*.

Upon exposure to NTZ and TIZ at either concentration, no effect on snail mobility or viability was observed up to 48 hours, when all snails were alive and showed normal activity. With NIC addition, snails became immobile within 2 hours, withdrawing into their shells. After 48 hours 25% of snails were dead at 0.5mg/litre and 45% of snails were dead at 1mg/litre. Therefore, neither NTZ nor TIZ has molluscicidal activity against the *S. mansoni* intermediate host *B. glabrata*.

4.13 Discussion.

Although NTZ has good activity against *Fasciola hepatica* in humans (Favennec *et al*, 2003) this trematode was not available to study. Therefore, the mechanism of action of NTZ against trematodes was examined using the blood-fluke *Schistosoma mansoni*. Both NTZ and TIZ showed clear activity against all stages of schistosomes apart from the eggs, characterised by rapid paralysis, shrinkage and tegumental damage. The drug effects were diminished in the presence of serum. Nutrient uptake was also affected in the presence of NTZ, and ATP levels were significantly lowered. BZNT, a related compound, was also active against schistosomes but the effects were distinct from those of NTZ.

4.13.1 Paralytic effect.

a) Without serum.

NTZ or TIZ (10μg/ml and 1μg/ml) caused a rapid curling of adult *S. mansoni* and paralysis; separation of pairs was followed by shrinkage and darkening of the tegument. The effects were comparable to those of PRAZ with similar potency and speed of paralysis development. However, NTZ and TIZ produced a quicker killing effect, with 100% schistosome mortality observed by 24 hours, whereas schistosomes exposed to PRAZ took up to 120 hours to die. BZNT also caused paralysis but with a slower onset, and this was not lethal within 120 hours.

In schistosomula, NTZ and TIZ, similarly at 10μg/ml, caused rapid paralysis and tegumental disruption which was more profound than that observed with PRAZ which acted only after about 3 hours. BZNT caused rapid paralysis and mortality occurred at lower concentrations than observed with NTZ. These differences suggest a difference between mechanisms of action of BZNT and NTZ.

The protonophores FCCP, CCCP and niclosamide showed very similar paralysis production in *S. mansoni* adults to that observed with NTZ and TIZ, characterised by shrinkage and curling. This suggests that a protonophore effect may be an important aspect of the mechanism of action of NTZ against schistosomes.

b) With serum.

Addition of 10% FCS to the medium significantly lowered activity of NTZ/TIZ against schistosomes. This is due to the drug having a high affinity for albumin with >99% bound to albumin in human blood. (Romark, Pharmaco-Toxicological expert report, 1999). FCS did not, however lower the activity of praziquantel presumably because of lesser binding to serum proteins: 20% of PRAZ was unbound (Vanden Bossche, 1985). NTZ was found to be inactive against *S. mansoni* in mice Q.D.Bickle (2000) (personal communication). Presumably this is related to the very low concentration of free NTZ / TIZ in the plasma.

4.13.2 Drug uptake.

The rapidity of NTZ effects on *S. mansoni* suggest that the drug is absorbed very rapidly. Since trematodes, unlike nematodes lack a cuticle, and actively absorb nutrients e.g. glucose through the tegument (Rogers & Bueding, 1975), it seems likely that the drugs are being absorbed directly rather than ingested. This is supported by the clear tegumental disruption after 1 hour NTZ exposure. Ingestion however, could also be occurring.

4.13.3 Tegumental damage.

By light microscopy NTZ caused blebbing and granulation of the schistosome tegument. Adult males were the most affected and were also more susceptible than females to lower concentrations of drug. Scanning electron microscopy also confirmed these observations and also showed desquamation, lesions and tegumental swelling. These effects were very similar to those of PRAZ (Apinhasmit & Sobhon, 1996) on the liver fluke *Opisthorchis viverrini.*

4.13.4 Glucose uptake.

NTZ or TIZ (10ug/ml) significantly inhibited uptake of glucose in adult *S. mansoni* or schistosomula. Rogers & Bueding, (1975) have shown that in *S. mansoni* glucose is actively taken up by the tegument and accumulates over time. Inhibition of glucose uptake in this study is therefore, probably associated with the severe damage of the tegument observed with NTZ exposure affecting active absorption. At a lower concentration of NTZ

(1μg/ml), glucose uptake was less affected, and correlated with the level of tegumental damage. However, owing to the speed in which paralysis and death occurred in the flukes, it is likely that another mechanism of toxicity causes the pathogenesis. PRAZ did not inhibit glucose uptake in adults which further suggests that nutrient transport is unrelated to the rapid paralysis produced by this drug. PRAZ did inhibit glucose uptake in schistosomula, and its slow toxic effect on this stage could be due to this.

4.13.5 NTZ effect at nicotinic receptor.

a) Nicotinic antagonists.

Effects of NTZ on nicotinic receptors were investigated using the same receptor antagonists as used for *C. elegans* (see chapter 3). Pre- or post-treatment with mecamylamine, pempidine or atropine neither prevented nor abolished, respectively, NTZ/TIZ induced paralysis in schistosomes. This is in agreement with previous studies by Day *et al*, (1996), in which mecamylamine was an ineffective antagonist of acetylcholine in *S. mansoni*. Presumably, this may be due to the structure of acetylcholine receptors being different in trematodes to those in nematodes thus preventing antagonistic action. However they were still similar enough to interact with NTZ.

b) Alpha-Bungarotoxin (BTX).

Since mutants/genetic methods are not available for schistosomes, the nicotinic-like acetylcholine receptors of the schistosome were examined directly using a specific protein toxin (rhBTX) which can be visualised binding to schistosomes (e.g. *S. haematobium,* Camacho, 1995). Nicotinic acetylcholine receptors (nicotinic AchRs) are ligand- gated ion channels that produce an increase in Na^+ and K^+ permeability, depolarization and excitation upon activation (Haughland, 1998). Alpha- bungarotoxin, binds with a high affinity to the α-subunit of the nicotinic acetylcholine receptors of neuromuscular junctions (Haughland, 1998). Molecular Probes supply several fluorescent α-bungarotoxin conjugates to facilitate visualisation of nicotinic AchRs and α-bungarotoxin with an attached fluorophore of tetramethylrhodamine was used to study possible nicotinic receptor interaction by NTZ. The wavelengths of excitation and emission of tetramethyl-rhodamine (both over 500nm),

were free from interference by the yellow NTZ and analogues, whose absorbance peaks are below 500nm.

RhBTX clearly binds to nicotinic receptors in *S. mansoni* and *S. japonicum* adults and schistosomula. Addition of NTZ or TIZ inhibited or abolished this binding. The inhibition of rhBTX binding by NTZ suggests that it competes with rhBTX for the receptor, leading to a displacement of rhBTX or prevention of binding of rhBTX. This suggests that NTZ is an agonist for the nicotinic AchR, identical to a likely primary mechanism of action of NTZ in *C. elegans* deduced from genetic analysis (Chapter 3). In schistosomula the rapidity of drug effect and evidence of surface layer degradation under phase contrast suggest a surface membrane-associated mode of entry by NTZ. The pattern of fluorescence observed in this study was different to that previously shown by Camacho & Agnew, (1995), owing to rhBTX incubation carried out at 37°C instead of cold medium which presumably prevented uptake of the reagent into the body. BZNT did not inhibit rhBTX binding in adults, but in schistosomula caused a chaotic pattern of fluorescence. This difference in pattern of fluorescence in the presence of BZNT, also supports that this drug may have a different mode of action to NTZ and suggests extensive damaging of the organism. BTX on its own, had no effect on worm motility and did not appear to prevent NTZ-related motility changes.

A control study was carried out using pirenzepine, a muscarinic agonist which binds to the M_1 muscarinic AchR in the visual cortex of mice (Wang, 1994). Red fluorescent Bodipy 558/568 was chosen as a fluorophore because of the similarity in wavelength to rhBTX. Bodipy pirenzepine showed a clear binding to muscarinic receptors in *S. mansoni* and this was unaffected by NTZ, indicating that NTZ does not inhibit binding of agents to neurotransmitter receptors generally. Although NTZ quenched the fluorescence of rhBTX significantly *in vitro*, it also quenched the fluorescence of BoP and, in spite of this, it did not prevent visualisation of the muscarinic receptors in *S. mansoni* adults.

4.13.6 NTZ protonophoric effect.

NTZ and TIZ significantly reduced ATP levels in adult *S. mansoni*. This reduction was comparable to the reduction caused by FCCP and niclosamide, and again suggests that NTZ has similar mechanism of action to these drugs. This reduction in ATP could explain the similarity in paralysis between these drugs as a decrease of ATP in the worm would affect nerve signal transduction via Ca / Na ATPase, hence causing rapid paralysis. This has already been suggested as the cause of paralysis seen in *Dipylidium caninum* by niclosamide (Sano *et al*, 1982). PRAZ at 10μg/ml did not significantly alter ATP levels in *S. mansoni*, however at the lower concentration of 1μg/ml, a significant decrease was seen. This again highlights the differences in action of NTZ and PRAZ.

4.13.7 NTZ as a molluscicide.

Comparison of NTZ and bayluscide (niclosamide) showed no molluscicidal activity against *B. glabrata* with the Romark compound. Previous studies with BZNT (Dreyfuss *et al*, 1996) have shown this compound to be effective against *Lymnaea glabra*, again suggesting a differing mechanism between the two compounds. NTZ however may perhaps have molluscicidal activity against this other species due to its structural similarity to BZNT.

4.13.8 Conclusions.

With trematodes, it appears a few mechanisms of action of NTZ/TIZ are in effect, including an effect at the nicotinic receptor, reduction of ATP levels caused by a possible protonophoric effect and an effect on nutrient uptake. Both drugs cause a rapid paralysis and killing effect similar to that seen with the known uncouplers FCCP, CCCP and niclosamide, but different to that seen with PRAZ suggesting a protonophoric mechanism. This is further supported with NTZ significantly reducing ATP levels in the fluke. NTZ also causes clear tegumental damage which probably serves to lower the amount of glucose uptake of the fluke. However this is unlikely to be the primary mechanism of action owing to the speed in paralysis and mortality of the worm being too fast to be directly induced by lack of nutrients. Some effect at nicotinic receptors in schistosomes was observed where

NTZ was seen to clearly inhibit BTX binding to the receptor. Comparison with the known uncoupler CCCP, showed no inhibition of rhBTX binding suggesting that NTZ-induced paralysis in schistosomes is due to drug interaction at the nicotinic receptors. However, uncoupling could still be a factor of paralysis (and reduction of glucose uptake, which needs ATP), as an ATP drop in the worm would affect nerve signal transduction via Ca / Na ATPase and would cause rapid paralysis this way. This would explain the similarity in paralytic effects seen with FCCP, CCCP and niclosamide. NTZ demonstrated no molluscicidal activity. BZNT showed some activity against schistosomes, but type and speed of paralysis and lack of mortality suggests a different mechanism to that of NTZ. The reason for this is that BZNT lacks a substituent on the aromatic ring rendering it more likely to be metabolised differently. Hydrolysis of the amide would yield the highly toxic amino nitrothiazole which is not formed from NTZ / TIZ.

CHAPTER 5 – CESTODES.

5.1 Introduction.

Cestodicidal activity of NTZ against human parasites was first described in a clinical study (Rossignol and Maisonneuve,1984). More recently, further clinical trials have shown good efficacy of NTZ against cestodes with an 82% cure rate of hymenolepiasis and a 98% reduction in egg production found in Peruvian children (Juan *et al*, 2002). *In vitro*, nitazoxanide (10μg/ml) caused rapid damage to the ultrastructure and cellular destruction of metacestodes of *Echinococcus multilocularis* (Stettler *et al*, 2003). In the present project, studies were carried out *in vitro*, using adult and cysticercoid stages of *Hymenolepis diminuta* and cysticercoid stages of *Hymenolepis microstoma*. Effects of NTZ on motility and morphology were compared with those on nematodes and trematodes. Cestodes were very susceptible to NTZ demonstrating rapid paralysis and death. Oxygen uptake in mitochondria was stimulated in the presence of NTZ and severe tegumental damage was observed. BZNT as observed with trematodes caused paralysis and tegumental damage in *Hymenolepis*, though effects were less severe than with NTZ. DNNTZ, unlike with nematodes and trematodes caused slight paralysis and some tegumental damage.

5.2 Activity of Romark drugs and control drugs against *H. diminuta.*

5.2.1 NTZ and TIZ: Effects on adult *H. diminuta.*

Single, freshly isolated adult *H. diminuta* were placed into 15ml of Earle's balanced salt solution, in a plastic Petri dish. NTZ or TIZ in DMSO were added at concentrations of 10μg/ml or 1μg/ml and the effects observed at ambient temperature for 4 hours using initially a bench dissecting microscope and then an inverted microscope (20x magnification). NTZ or TIZ at either 10 or 1μg/ml immediately increased the motility of the adult tapeworms, followed by visible spastic contractions starting from the scolex, running through the neck and then along the entire length of the strobila. After approximately 20 minutes the contractions ceased and the worms

began to curl up and became paralysed in a similar way to that observed with *S. mansoni* (Chapter 4).

Paralysis was tonic with no worm movement evident, and after 1 hour, tegumental damage (characterised by blebbing) to the neck and proglottid segments was observed under an inverted microscope. Scolex integrity was also clearly affected and characterised by swelling. Worms appeared dead after 3-4 hours.

If worms were exposed to 10μg/ml NTZ or TIZ for 1 hour and then the drug was removed, death still occurred within 4 hours showing that drug effect was irreversible at this concentration and time. Unfortunately, concentrations of NTZ below 1μg/ml were not tested (and hence LD_{50}'s not found) due to a lack of tapeworms. The diluent DMSO (0.2%) had no effect on worm motility or viability and no visible effect on worm tegument up to the assay end point of 4 hours.

5.2.2 Effect of other Romark compounds with adult *H. diminuta.*

On addition of 10μg/ml DNNTZ or 10μg/ml BZNT, effects were initially similar to those seen with 1 or 10μg/ml NTZ, and adult worms within 5 minutes were demonstrating slight spastic contractions running along the length of the worm's body. However, the contractions, were not as severe as those seen with NTZ/ TIZ and only lasted for 5-10 minutes. After 10 minutes the contractions ceased and worms in either DNNTZ or BZNT partially recovered. No tegument damage was observed with either drug, and movement (though sluggish) was still evident after 4 hours. Concentrations of 1μg/ml DNNTZ and 1μg/ml BZNT showed some slight contraction of the worm body, but paralytic effects were not clearly observed. TIZg had no effect on the motility or morphology of adult *H. diminuta* at 1μg/ml or 10μg/ml.

5.2.3 Effect of praziquantel with adult *H. diminuta.*

Addition of 10μg/ml PRAZ to adult *H. diminuta* caused paralysis and contraction was observed after 10 minutes. The contraction was tonic, but was not as severe as the spastic contractions observed with NTZ and TIZ. After 4 hours worms were still paralysed but no mortality was observed. After 4 hours no damaging effect on worm tegument was observed and the worm scolex appeared unaffected.

Table 5.1 Visual effects of NTZ and other compounds on adult *H. diminuta* after 4 hours.

Compound (10μg/ml)	Paralysis	Death	Tegument damage	Scolex damage
NTZ	Yes	Yes	Yes	Yes
TIZ	Yes	Yes	Yes	Yes
PRAZ	Yes	No	No	No
BZNT	Yes	No	No	No
DNNTZ	Yes	No	No	No
TIZg	No	No	No	No

Table 5.1: Adult *H. diminuta* were placed into 15ml of Earle's balance salt solution, in a plastic petri dish and drug added to a final concentration of 10μg/ml. Effects were monitored at ambient temperature and included paralysis, tegument damage scolex damage and death. Effects with NTZ and TIZ were the most severe causing paralysis and death. PRAZ, DNNTZ and BZNT caused paralysis but did not cause death. TIZg had no effect. Molar concentrations used for each drug were as follows: NTZ (33μM), TIZ (38μM), PRAZ (32μM), BZNT (40μM), DNNTZ (38μM) and TIZg (23μM)

5.3 Effect of drugs on excysted protoscolices of *H. diminuta* and *H.microstoma.*

In order to examine NTZ related effects on the larval stages of *Hymenolepis* spp, cysticercoids of either *H. diminuta* or *H. microstoma* were excysted and a suspension in Dulbecco's Modified Eagle's medium (DMEM) divided into wells of a 96 well plate (100μl) then drug was added in triplicate assays from 0.1μg/ml to 30μg/ml. Worms were incubated at 37°C and monitored microscopically using an inverted microscope (40x magnification) for a period of 4 hours.

5.3.1 Effects with *H. microstoma.*

5.3.1.1 Effects with NTZ and TIZ.

Excysted cysticercoids were fully motile in DMEM and demonstrated scolex elongation and sucker movement. Addition of 10µg/ml NTZ or TIZ to excysted cysticercoids of *H. microstoma* caused an immediate reaction, characterised by paralysis of the scolex causing motility and sucker movement to cease. Within a few seconds blebbing started to occur, the suckers retracted and the rostellum opened revealing damage in the formation of the hooks. This was followed by an enlargement of the scolex until integrity was lost and total collapse occurred within 5-10minutes (see figure 5.1b). This form of disintegration was seen in 67% of excysted cysticercoids at this concentration, with the remainder becoming paralysed and showing no movement or other activity within 30 minutes. At 1µg/ml the effect of either drug was slower and only 20% of scolices showed disintegration within 30 minutes. After 30 minutes all non-disintegrated scolices were paralysed but still alive, but after 1 hour all scolices were moribund. No recovery from exposure to 1µg/ml NTZ or TIZ was observed. After 24 hours, all cysticercoids exposed to 1µg/ml NTZ or TIZ had disintegrated. No difference in paralysis or speed of effect was observed between NTZ and TIZ at any concentration tested. Addition of 0.1µg/ml NTZ or TIZ had no effect on cysticercoid motility or morphology: 100% of worms were alive and fully active after 24 hours. Equal concentrations of the drug diluent DMSO had no effect on scolex motility or integrity. Table 5.2 shows the activity of varying concentrations of NTZ on the motility of *H. microstoma* excysted cysticercoids during a 24 hour period.

Table 5.2 NTZ induced effects on the motility/activity of excysted cysticercoids of *H. microstoma* over time.

Conc/time	10 mins	30 mins	1 hour	2 hours	4 hours	24 hours
10µg/ml (33µM)	0 (67)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
5µg/ml (16µM)	0 (50)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
1µg/ml (3.3µM)	3	2 (20)	1 (20)	1 (20)	1 (20)	0 (100)
0.8µg/ml (2.6µM)	4	3	3	2	2	0 (100)
0.4µg/ml (1.3µM)	4	4	3	3	2	0 (100)
0.1µg/ml (0.33µM)	4	4	4	4	4	4

Key to table 5.2

Activity of cysticercoid. Parentheses show percentage mortality.

4 = Good activity
3 = Sluggish
2 = paralysed
1 = Moribund
0 = Dead

Table 5.2: Effect of varying concentrations on the motility of cysticercoids of *H. microstoma* over 24 hours. Examination was carried out using an inverted microscope at 40x magnification. Between microscopic examinations, cysticercoids were placed in a 37°C incubator in 95%air/5% CO_2.

Excysted scolices exposed to 10µg/ml NTZ or TIZ were also removed from drug and placed in fresh media after 30 minutes. No recovery however was observed and worms became moribund and died as above.

5.3.1.2 Effect of other Romark compounds on excysted protoscolices of *H. microstoma.*

Addition of 10µg/ml DNNTZ and BZNT to excysted cysticercoids caused a similar but less severe effect to that observed with NTZ and TIZ. Scolices on exposure to either

compound became paralysed within 5 minutes and sucker movement was reduced. However, the extreme disruption observed with NTZ or TIZ, was not evident. After 30 minutes exposure to DNNTZ, scolices showed some recovery (though motility was not back to the level of the control) and no death was observed. Scolices exposed to BZNT did not show any recovery and all cysticercoids were moribund after 1 hour. Scolices exposed to 1µg/ml DNNTZ showed no drug effect, whereas 1µg/ml BZNT did cause motility impairment though much reduced from that observed in the higher concentration, with paralysis taking several hours to occur. Death was not observed with DNNTZ at either concentration up to 24 hours, whereas with BZNT scolex disintegration was observed at either 1 or 10ug/ml. TIZg had no effect on cysticercoid motility or morphology up to 30µg/ml after 24 hours.

5.3.1.3 Effect of other drugs on excysted protoscolices of *H. microstoma.*

Addition of 10µg/ml NIC, FCCP or CCCP, caused rapid paralysis in the exposed cysticercoids similar to that observed with NTZ. After 1 hour all sucker movement had ceased and clear damage to the tegumental integrity was observed characterised by collapse. At 1µg/ml onset of paralysis with each drug occurred much more slowly, taking up to 1 hour to become evident. At four hours post drug addition all cysticercoids demonstrated damage to the scolex and all three drugs at 1µg/ml caused complete scolex disintegration by 24 hours post-exposure.

Addition of 10µg/ml praziquantel caused paralysis, but at a much slower rate than observed with NTZ, NIC, FCCP or CCCP. However all cysticercoids were paralysed within 4 hours, though no damage to the scolex was observed. After 24 hours all cysticercoids were alive but clear granulation and scolex damage was evident. At 1µg/ml, PRAZ failed to cause paralysis of cysticercoids after 4 hours. After 24 hours all cysticercoids were alive and motile, however some scolex damage and granulation was again evident. Addition of 10µg/ml MET or LEV had no effect on worm motility or morphology.

Figure 5.1 Infective excysted scolices of *Hymenolepis microstoma* in the presence of a) DMSO and b) 10µg/ml NTZ.

a)

b)

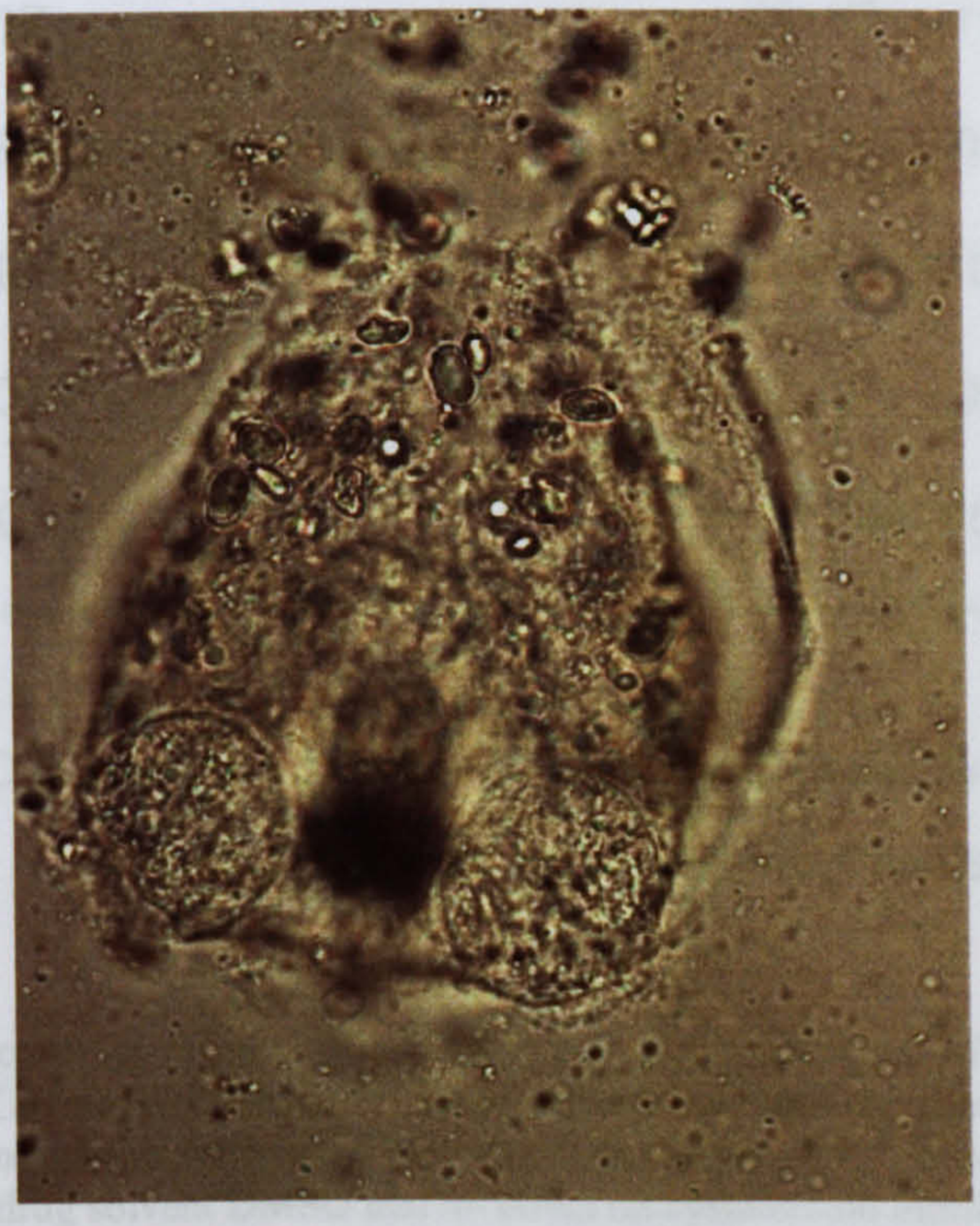


Fig 5.1: Cysticercoids of *H .microstoma* were exposed to 10µg/ml of NTZ for 10 minutes in DMEM at 37°C. After 5 minutes cysticercoids became paralysed and scolex integrity failed fig 5.1 b), causing it to burst. An equal concentration of the drug solvent DMSO had no effect on cysticercoid motility or scolex integrity, fig 5.1 a).

5.3.1.4 Quantitative comparison of sensitivity of *H. microstoma* cysticercoids to NTZ and other compounds in DMEM in the absence of FCS.

Since larger numbers of *H. microstoma* cysticercoids were available, the effects of NTZ on motility was tested at several concentrations for 4 hours and the MI_{50} (concentration to affect motility in 50% of worms) determined. It was not possible to quantitate the cestodicidal activity owing to difficulty in discerning parasite death.

Table 5.3 Effect of Romark compounds and control drugs on *H. microstoma* motility: quantitative data.

Compound	MI_{50} ± S.E. (μM)
NTZ	0.55 ± 0.2
TIZ	0.58 ± 0.1
DNNTZ	< 19.0 > 3.8
BZNT	< 8.0 > 4.0
TIZg	(No effect at 68μM)
PRAZ	2.9 ± 0.7
NIC	0.61 ± 0.5
FCCP	0.7 ± 0.3
CCCP	0.44 ± 0.1

MI_{50} = Concentration of drug which results in motility inhibition in 50% of the worm population in 4 hours.

Table 5.3: Cysticercoids of *H. microstoma* were exposed to 10μg/ml of drug for 4 hours in DMEM (without FCS) at 37°C. After 4 hours, effects on motility characterised mainly by cessation of sucker movement were noted and MI_{50}'s calculated using Sigmaplot 2000. An equal concentration of the drug solvent DMSO had no effect on cysticercoid motility within 4 hours.

5.3.1.5 Effects of drugs on *H. microstoma* cysticercoids in the presence of serum.

Addition of 10% FCS dramatically lowered the potency of NTZ/TIZ against *H. microstoma* cysticercoids. Addition of 10μg/ml NTZ/TIZ caused sluggishness within 10 minutes, followed by paralysis, but scolex integrity was unaffected, and all cysticercoids were alive after 4 hours. In 1μg/ml NTZ/TIZ all cysticercoids were fully motile, demonstrating normal sucker movement compared with the solvent control even after 24 hours. The MI_{50}'s of NTZ and TIZ were increased more than 10-fold to 7.0 ± 0.6μM and 8.4 ± 1.1μM respectively. Addition of serum had no effect on the potency of PRAZ, CCCP, FCCP or niclosamide: MI_{50}'s did not differ significantly from those observed when serum was absent.

5.3.2 Effect of NTZ on cysticercoids of *H. diminuta.*

5.3.2.1 Effects with NTZ and TIZ.

With addition of 10μg/ml NTZ or TIZ to *H. diminuta* complete paralysis was observed in 100% of cysticercoids within 5 minutes, characterised by lack of motility and cessation of sucker movement. Drug effects however, were not as severe as those observed with *H. microstoma* cysticercoids and rapid loss of scolex integrity was not a factor. However, *H. diminuta* cysticercoids appeared dead within one hour of drug exposure and no recovery was observed. After 24 hours scolex disintegration had occurred in 100% of exposed cysticercoids. In 1μg/ml of NTZ/TIZ, paralysis took approximately one hour to occur and all cysticercoids became moribund within four hours. Again after 24 hours, all cysticercoids exposed to 1μg/ml NTZ or TIZ had disintegrated. With additions of NTZ and TIZ at 0.1μg/ml concentration, no effect on motility, sucker movement or morphology was observed, and all cysticercoids showed normal movement after 24 hours. Equal concentrations of the DMSO solvent control to those found in the drugged wells had no effect on scolex motility or morphology. All cysticercoids displayed normal motility after 24 hours.

5.3.2.2 Effect of other Romark compounds on excysted protoscolices of *H. diminuta.*

Addition of 10μg/ml DNNTZ or BZNT caused paralysis as rapidly as a similar concentration of NTZ/TIZ but was less severe, with some movement of suckers still evident. After 4 hours drug exposure, BZNT-treated worms were moribund, whereas those exposed to DNNTZ continued to show limited sucker movement. After 24 hours

BZNT exposed worms had disintegrated whereas those exposed to DNNTZ still showed signs of life. Addition of 1μg/ml BZNT again caused paralysis of cysticercoids though speed of paralysis was slower and scolices did not become moribund within 4 hours. Death was again observed after 24 hours. Addition of 1μg/ml DNNTZ merely induced a slowing of motility with cysticercoids appearing sluggish and no mortality was observed after 24 hours. TIZg, similarly to equal concentrations of the drug diluent DMSO had no effect on cysticercoid motility or morphology.

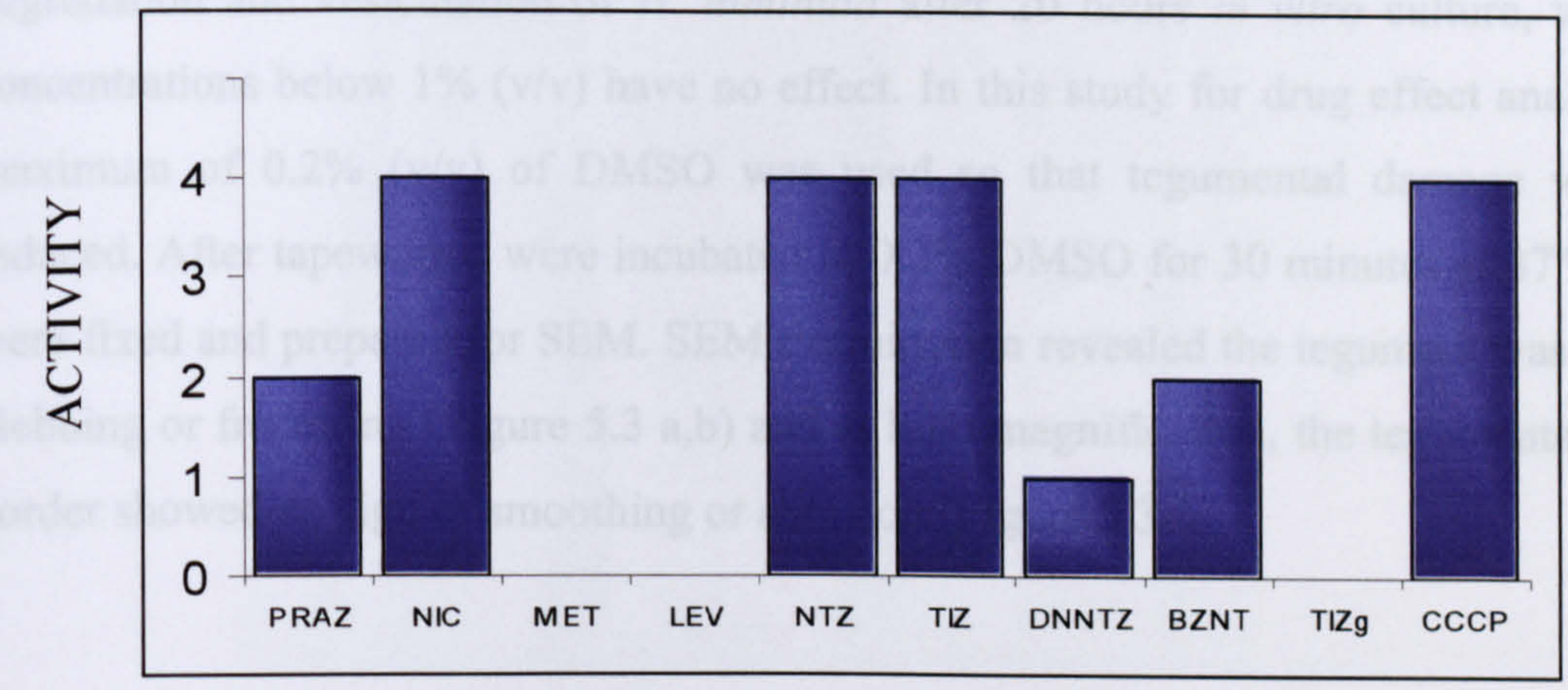
5.3.2.3 Effect of known anthelmintics and other compounds.

Addition of 10μg/ml NIC, CCCP or FCCP caused rapid paralysis of cysticercoids, indistinguishable from that observed with NTZ or TIZ, characterised by lack of motility and cessation of sucker movement. All cysticercoids appeared dead 1hour after drug exposure. All cysticercoids were dead and showed disintegration after 24 hours drug exposure. With 1μg/ml the same effects were observed except that onset of paralysis occurred after between 2-3 hours for each drug.

Praziquantel (10μg/ml and 1μg/ml) showed lesser effects exactly as observed with *H. microstoma*. Similarly, addition of 10μg/ml MET or LEV had no effect on motility or morphology of *H. diminuta* cysticercoids.

MI_{50}'s for NTZ/TIZ effect on *H. diminuta* cysticercoid motility were the same as observed with *H. microstoma*. Similarly MI_{50}'s with FCCP, PRAZ and NIC were the same for both species. Again addition of FCS (10%), reduced NTZ/TIZ activity.

Figure 5.2 Comparison of activity of Romark compounds and known anthelmintics against *H. diminuta* and *H. microstoma* cysticercoids.



Drug activity is defined as: 0 Not Effective. 1 Slight Effect.
2 Moderate Effect. 3 Strong Effect (Killing effect within 4 hours)
4 Very Strong Effect (Killing effect within 1 hour)

Fig: 5.2: Ten cysticercoids of *H. diminuta* or *H. microstoma* were exposed to 10µg/ml of drug in DMEM *in vitro* for 4 hours at 37°C in the absence of serum. NTZ/TIZ showed very strong activity against the cysticercoids and caused 100% mortality within 4 hours. The initial effect on *H .microstoma* was more severe than that observed with *H. diminuta* and involved rapid disintegration of the scolex. NIC and CCCP showed very strong activity and 100% of worms were moribund within 4 hours. PRAZ and BZNT had a moderate effect causing lack of motility. Unlike with nematodes and trematodes, DNNTZ induced some paralysis in both species. TIZg, metrifonate and the anti-nematodal drug LEV had no effect on cysticercoids of either species. The solvent control DMSO had no effect on cysticercoid motility or integrity.

5.4 Examination of morphological effects on cestodes.

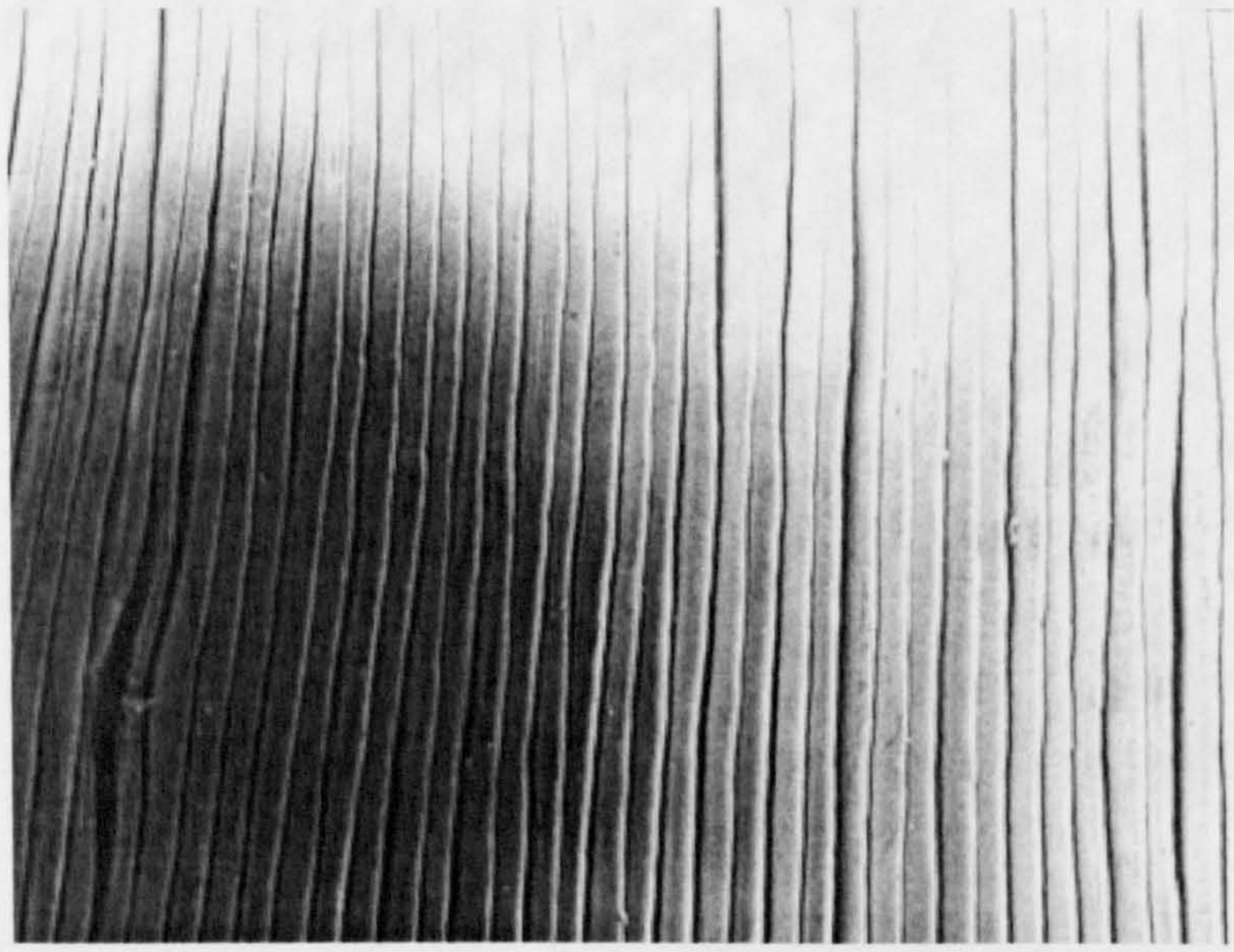
Previous studies have shown that anthelmintics can cause drug-induced pathomorphological changes in the tegument of tapeworm species e.g. taenifugin with *Hymenolepis fraterna* (Stoitsova *et al*, 1992) and praziquantel with *Bothriocephalus acheilognathi* (Pool, 1985). SEM studies were therefore carried out on samples of NTZ-treated rat tapeworm, *H. diminuta*. Treatments with DNNTZ or BZNT were also carried out for comparison.

5.4.1 Solvent control

Foreman & Oaks (1991) showed that DMSO above 1% (v/v) caused tegumental degradation and vesiculation of *H. diminuta* after 20 hours *in vitro* culture, whereas concentrations below 1% (v/v) have no effect. In this study for drug effect analyses, a maximum of 0.2% (v/v) of DMSO was used so that tegumental damage was not induced. After tapeworms were incubated in 0.2% DMSO for 30 minutes at 37°C, they were fixed and prepared for SEM. SEM examination revealed the tegument was free of blebbing or fracturing (Figure 5.3 a,b) and at high magnification, the tegumental brush border showed no sign of smoothing or abrasion (Figure 5.3c).

Figure 5.3 SEM analysis of solvent control-treated **tegumental surface of *H. diminuta*.**

a)

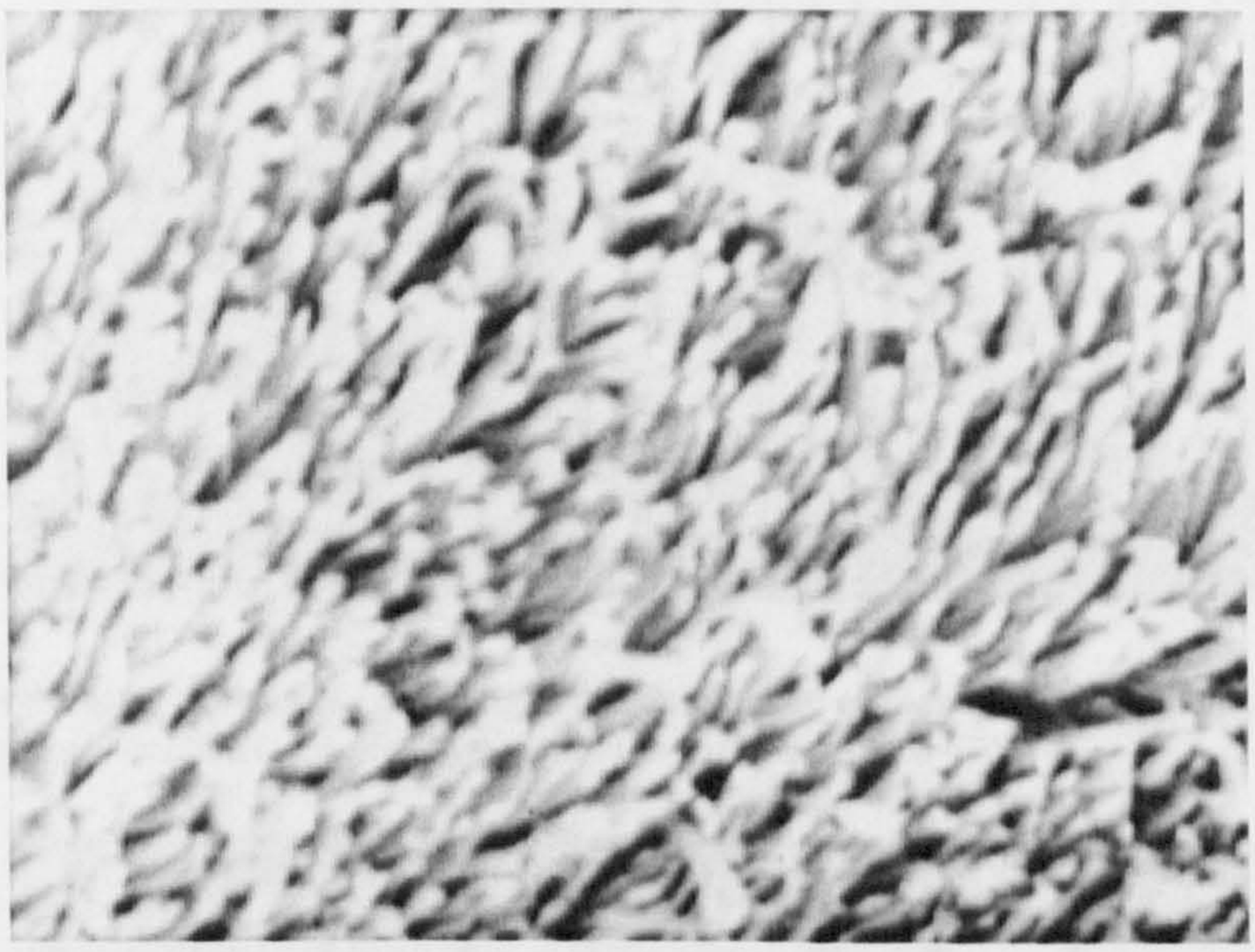


a) At magnification x70, no blebbing is evident and the worm segments seem less swollen than those that were drug-treated. This suggests that addition of drug causes some stretching of the worm.

b)

No fracturing of the tapeworm tegument is observed. Magnification x 700.

c)



Tegumental brush border appears intact with no sign of smoothing or abrasion. Magnification x15000.

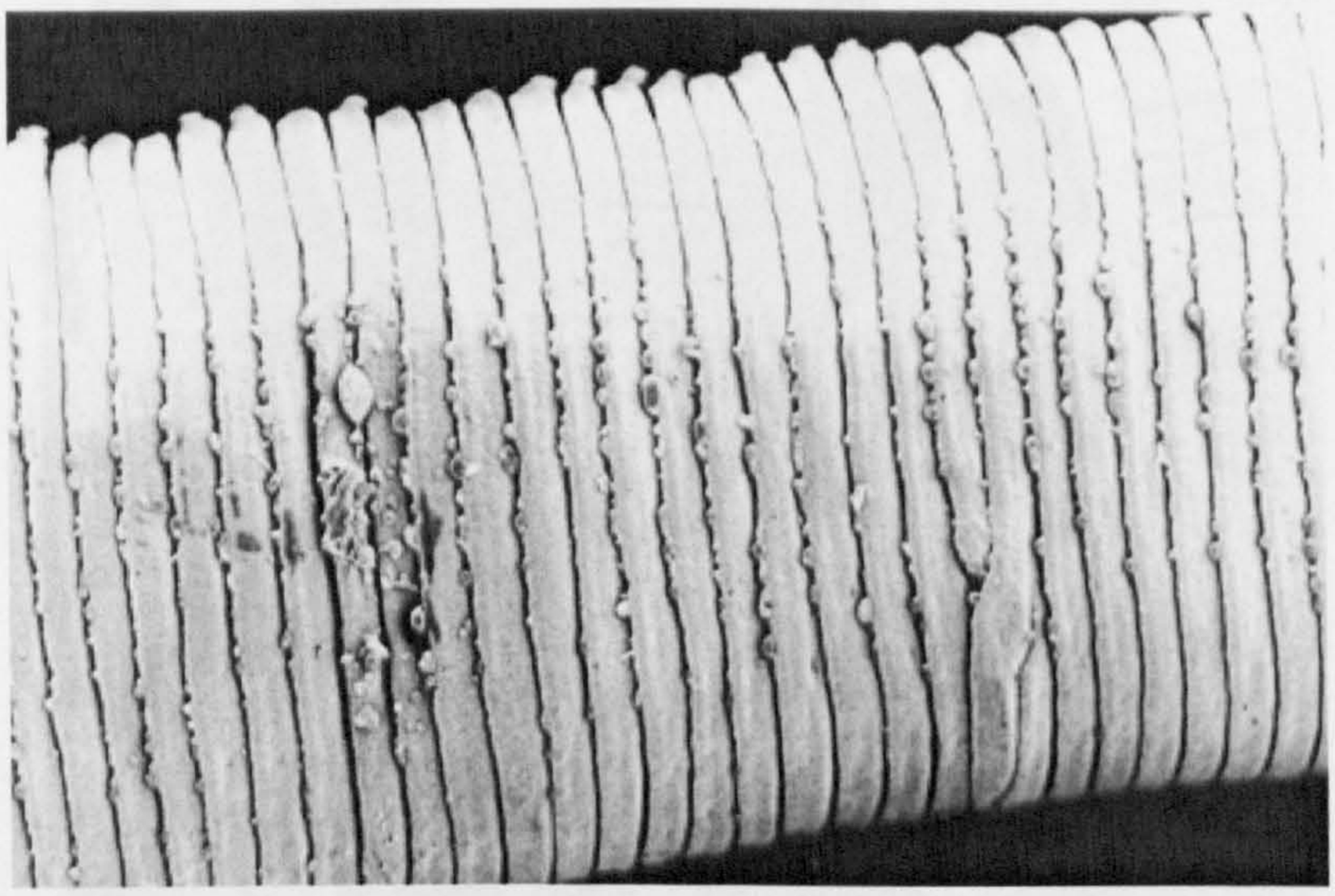
Figure 5.3: Adult *H. diminuta* were exposed to 0.2 % DMSO for 30 min at 37°C and effect on tegument examined using SEM. Magnification 70x , 700x and 15000x in a-c respectively.

5.4.2 Effect of NTZ.

NTZ caused clear damage to the tegument of *H. diminuta*. On the tegumental surface, there was blebbing as well as some accumulation of membrane fragments over the microthrix tips (figure 5.4 a, b, c and d) and a slight erosion of the tegumental brush border (Figure 5.4 f). The ultra structure of the tegument appeared swollen possibly due to drug-induced stretching, and fractures of variable depth were also visible (Figure 5.4c). This damage would probably produce an altered tegumental integrity and would cause disruption of the selective permeability barrier created by the normal tegument.

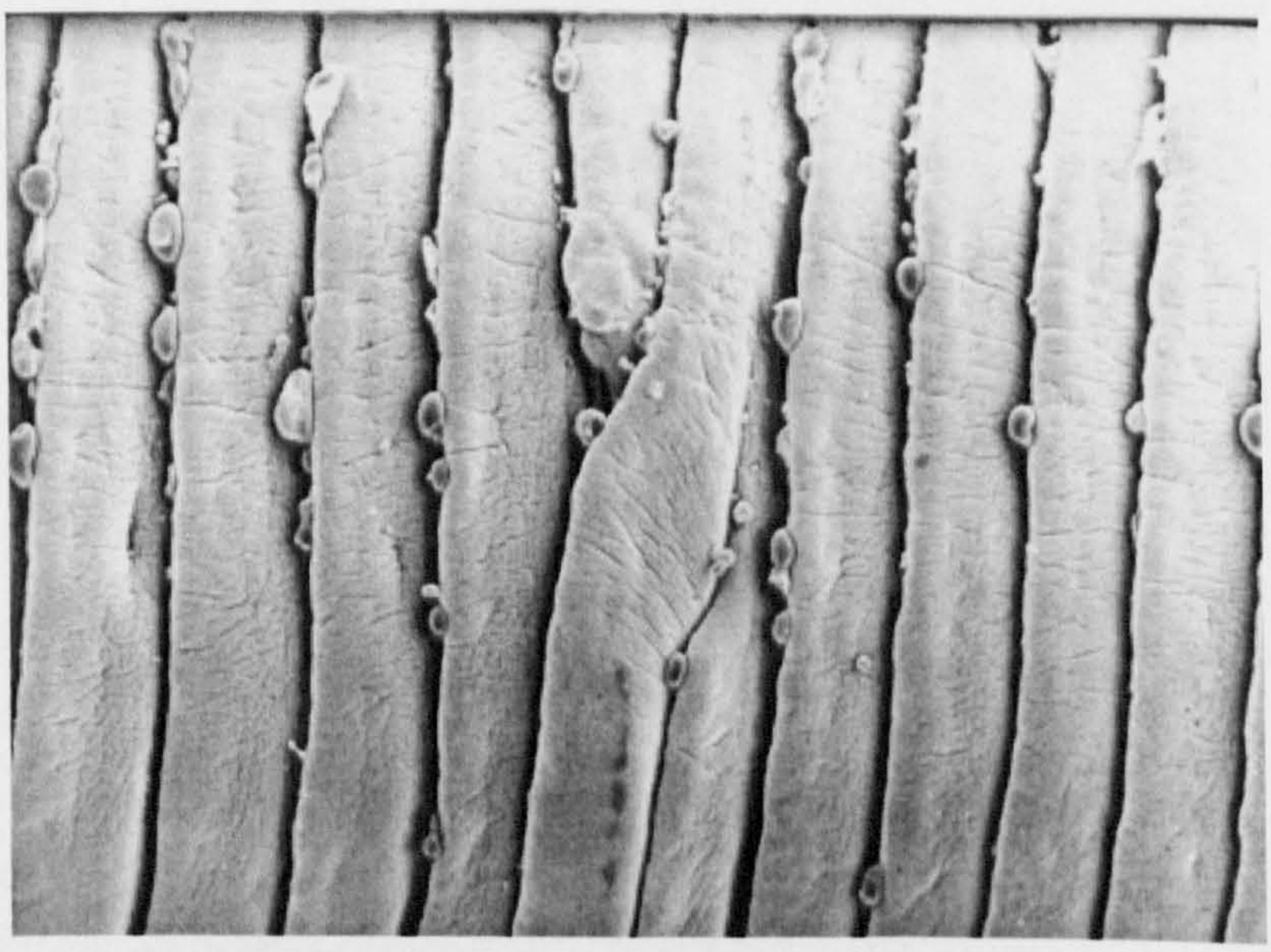
Figure 5.4 NTZ induced damage of *H. diminuta* tegumental surface.

a)

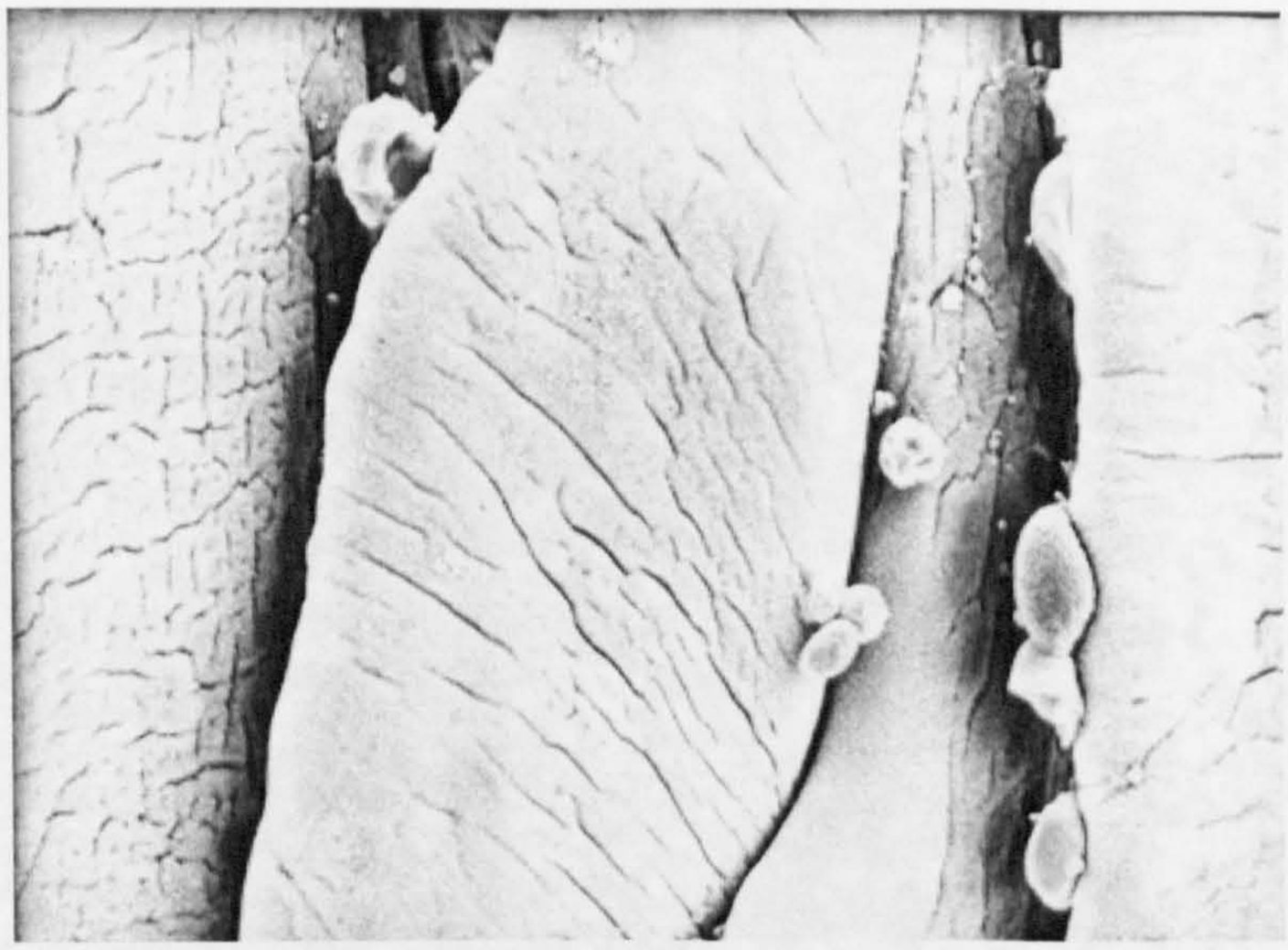


a) Magnification x70. Blebbing and accumulation of membrane fragments are clearly evident on the tapeworm surface due to presence of NTZ.

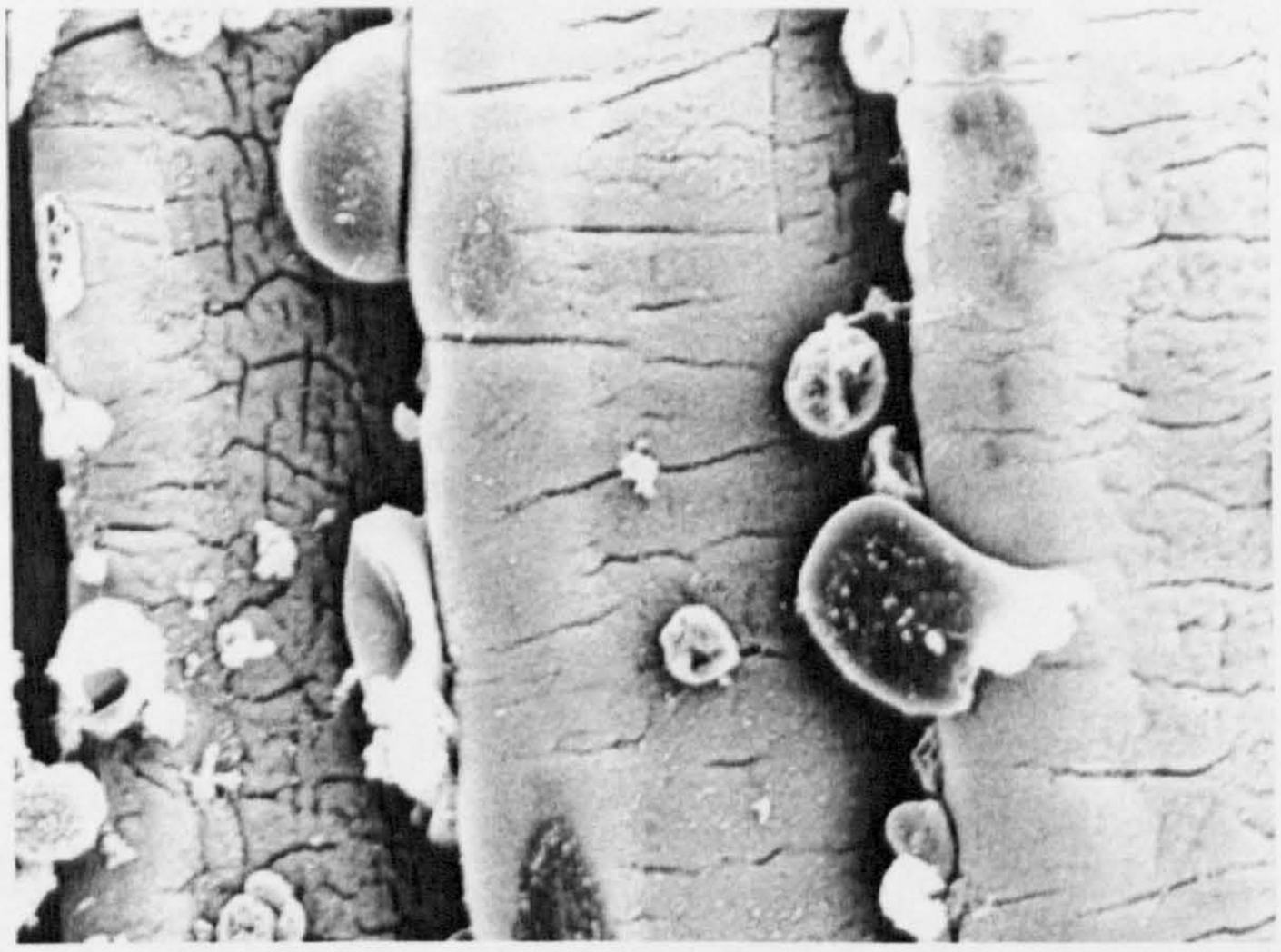
b)



c)



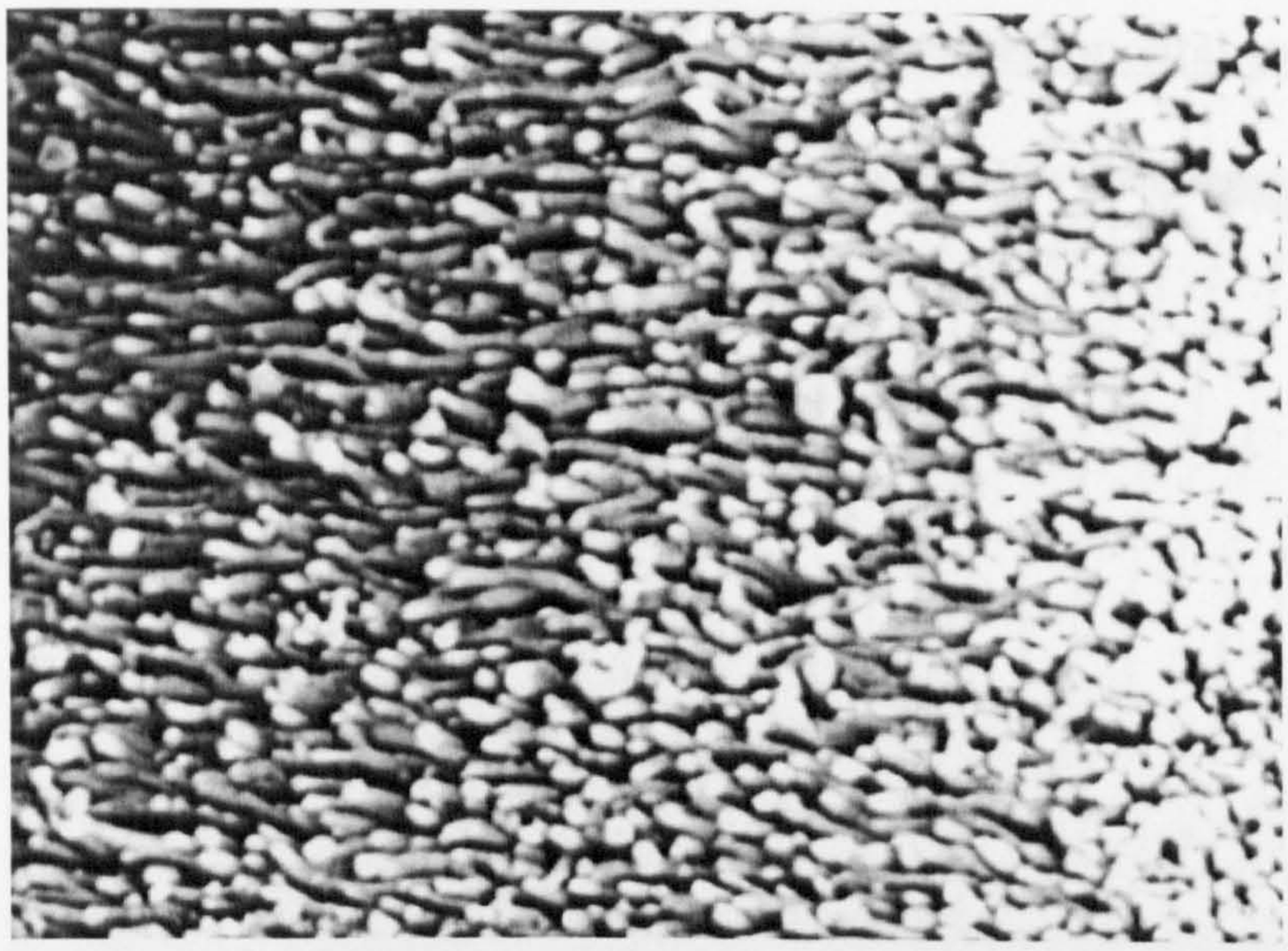
d)



b) Magnification x200 and c) & d) Magnification x700 show clear cracking and abrasion of the tegument surface due to NTZ. Segments are enlarged and swollen and damage to the ultra structure is seen. Blebs are evident.

e)

f)



e) Magnification x4500 shows cracking in the proglottid. f) magnification x 15000 shows smoothing of the tegumental brush border.

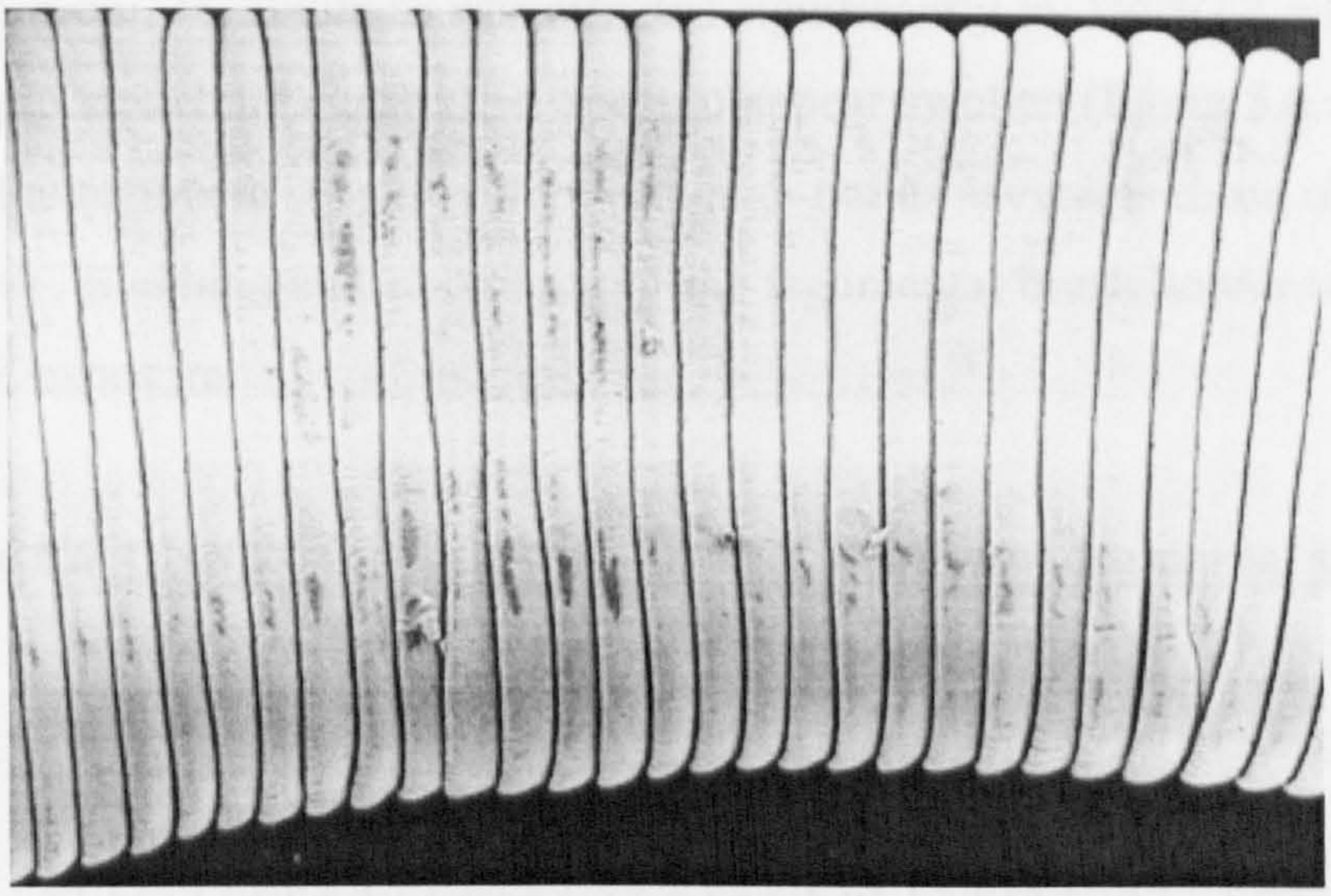
Figure 5.4: Adult *H. diminuta* were exposed to 10μg/ml NTZ for 30 min at 37°C and drug-effect on tegument examined using SEM. Magnifications are 70x, 200x, 700x, 700x, 4500x and 15000x for a),b),c),d),e) and f) respectively.

5.4.3 Effect of BZNT.

Addition of 10μg/ml BZNT did not cause any visible blebbing though some accumulation of membrane fragments on the tegument was observed (figure 5.5a). However, as with NTZ, some swellings due to possible worm expansion were present. Fractures on the tegumental surface were also observed (figure 5.5 b), which in some cases appeared more severe than those due to NTZ treatment. BZNT did not cause smoothing of the brush border.

Figure 5.5 BZNT induced damage of *H. diminuta* tegumental surface.

a)



b)

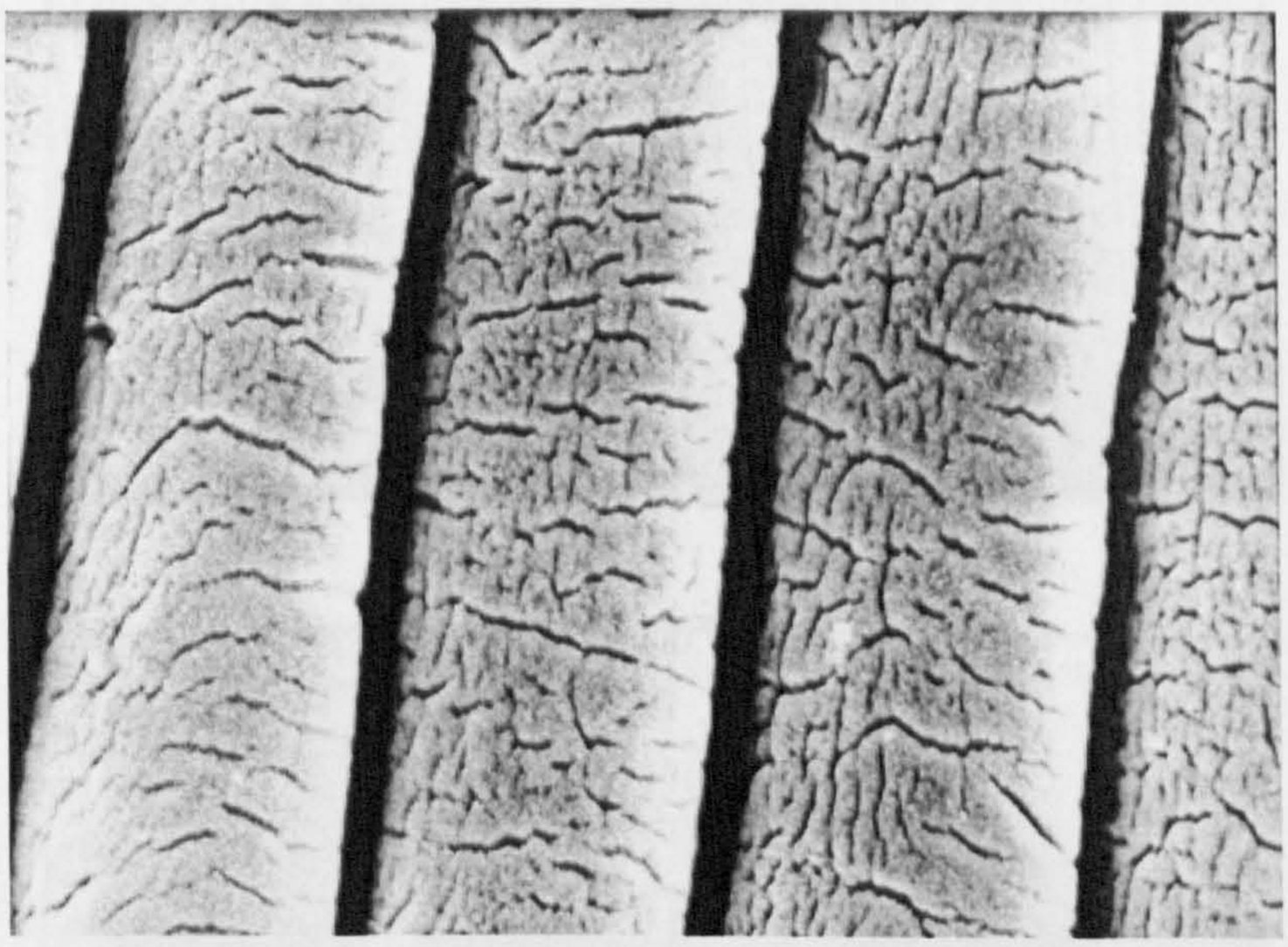


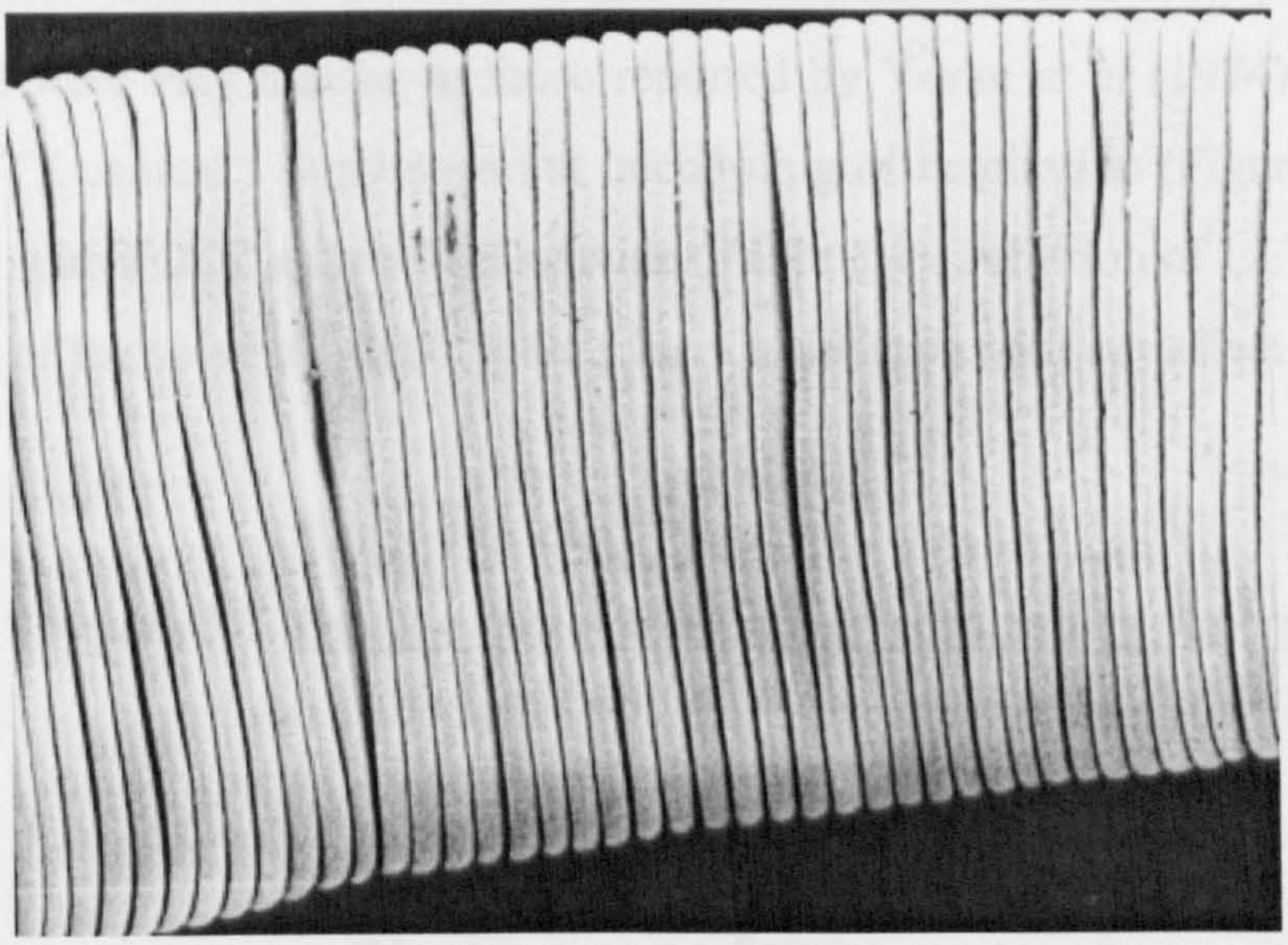
Figure 5.5: Adult *H. diminuta* were exposed to 10μg/ml BZNT for 30 min at 37°C and effects on the tegument examined using SEM. Addition of BZNT showed clear tegumental effect characterised by a) swelling and some membrane fragment accumulation at 70x magnification and b) extensive cracking at 700x magnification. Blebbing was not apparent, and the brush border was unaffected by the compound according to analysis at 15000x magnification (not shown).

5.4.4 Effect of DNNTZ.

Unlike *S. mansoni*, *H. diminuta* was affected significantly by DNNTZ when analysed by SEM. Slight stretching caused the worm to appear swollen (figure 5.6 a) with some fractures of the tegument (figure 5.6 b) although not as severe as those observed with NTZ or BZNT. Blebbing and smoothing of the tegumental brush border were not seen with DNNTZ exposure.

Figure 5.6 DNNTZ induced damage of *H. diminuta* tegumental surface.

a)



b)

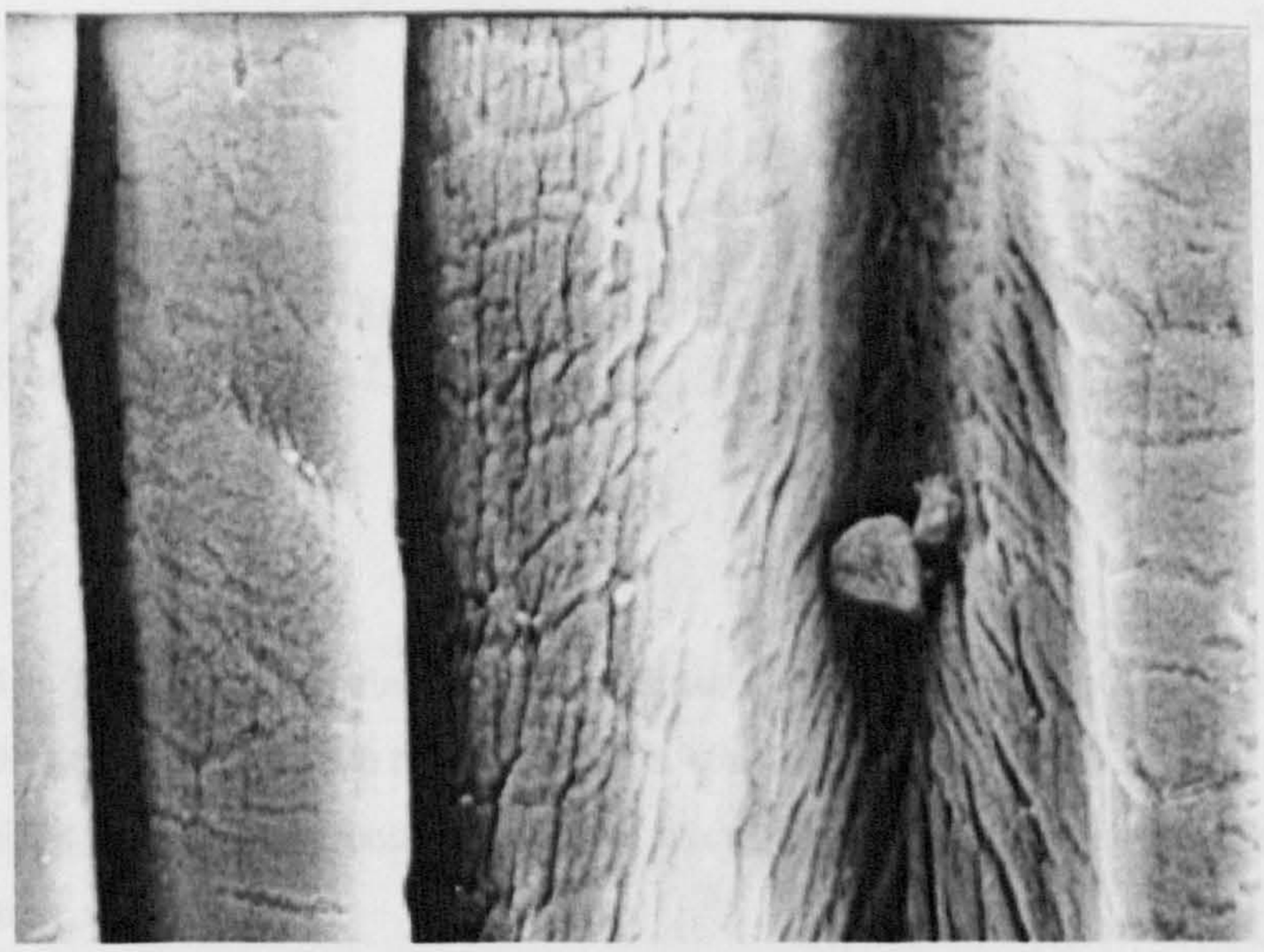


Figure 5.6: *H. diminuta* adults were exposed to 10µg/ml DNNTZ for 30 minutes and examined by SEM. Slight swelling but no sign of blebbing was observed at 70x magnification in a). Drug-induced fractures were seen at 700x magnification in b), though not to the extent as those seen with NTZ and BZNT. (The brush border examined at 15000x magnification was unaffected by DNNTZ, not shown)

5.5 Effects of NTZ/TIZ and selected uncouplers on the oxygen uptake of *H. diminuta* mitochondria.

In order to test whether NTZ was acting as a protonophoric uncoupler in *H. diminuta*, mitochondria were prepared, and effects on respiration examined using an oxygen electrode. The methodology was the same as that used to discern uncoupling by niclosamide and other compounds in this organism (Yorke *et al*, 1974).

Using 7.5mM succinate as respiratory substrate, addition of 200μM ADP showed a modest respiratory control in the *H. diminuta* mitochondria (Table 5.4). The respiration rates obtained were very similar to those reported by Yorke *et al* (1974). Addition of 10ug/ml of NTZ caused a rapid apparent uncoupling of respiration (Figure 5.7), which was also seen with FCCP and or Niclosamide (Table 5.4). Addition of LEV or an equal concentration of the solvent DMSO to that used in the drugs did not affect the uptake of oxygen.

Figure 5.7 Oxygen traces illustrating respiratory control.

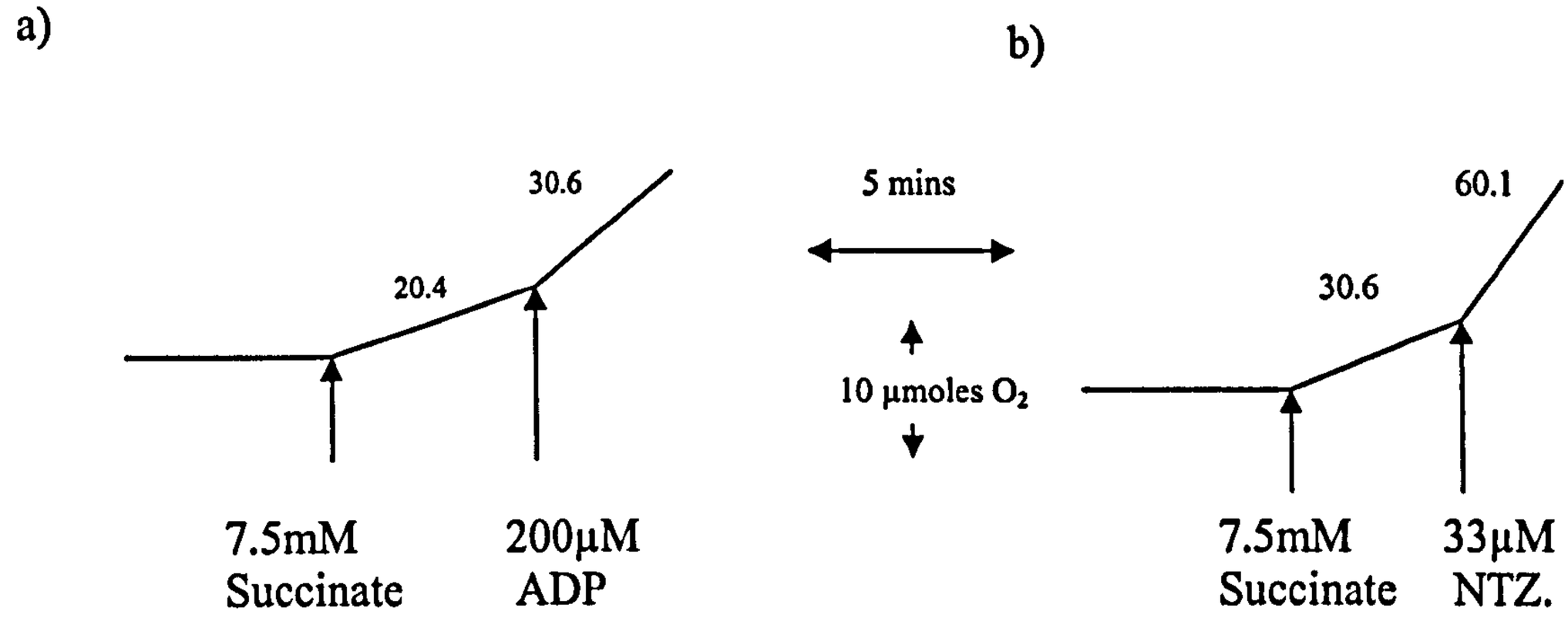


Fig 5.7: Typical oxygen electrode traces illustrating respiratory control, and the effect of a)ADP and b) NTZ on the respiration of *H. diminuta* mitochondria. Arrows indicate the additions and final concentrations. Respiration rates are in ng-atom of oxygen/min/mg protein.

Table 5.4 Effect of NTZ on Oxygen uptake of *H.diminuta* mitochondria.

Substrate	O_2 uptake (ng-atom/min/mg protein)						Respiratory control ratio.
	- ADP	+ ADP	+ NTZ	+ TIZ	+ FCCP	+ NIC	
Succinate	20.4	30.6					(1.5)
Succinate	30.6	61.1					(2.0)
Succinate	34.6		55.0				(1.6)
Succinate	40.7			71.3			(1.8)
Succinate	18.3				30.6		(1.7)

Table 5.4: Mitochondria were prepared from 5g (wet weight) of adult *H. diminuta* and respiration examined in the presence of 7.5mM sodium succinate in a 1ml stirred cell fitted with an oxygen electrode as described by Yorke *et al* (1974). Respiratory control was determined by addition of 200μM ADP, and uncouplers were added in DMSO, which had no effect on respiration when added alone. Results are the mean of 2 assays (due to limited material). Variability in succinate rate is due to the low rate measured which is more affected by oxygen leakage into the cell.

5.6 Discussion.

The mechanism of action on cestodes of NTZ, TIZ and other Romark compounds was examined using *Hymenolepis diminuta* and *Hymenolepis microstoma*. The results are fairly similar to those obtained with the trematodes characterised mainly by rapid paralysis and generally more obvious and lethal effects than those seen with nematodes. NTZ, BZNT and DNNTZ were found to have a damaging effect on the tegument of the tapeworm, suggesting a direct tegumental mechanism of action. NTZ also stimulated oxygen uptake in *H. diminuta* mitochondria, supporting its proposed protonophore activity (D.C. Warhurst & D.J. Meyer, 2000, Unpublished data)

5.6.1 Paralytic effects.

NTZ caused a very rapid (< 5 mins) and severe paralytic reaction in *Hymenolepis* leading to death. This effect was similar in appearance and occurred with similar potency to that associated with FCCP, CCCP or niclosamide, suggesting that it is due to a protonophoric mechanism. Praziquantel, though also inducing paralysis had a much lesser cestodicidal effect than NTZ/TIZ or the other protonophores suggesting that, as with trematodes, NTZ has a different mechanism to PRAZ. NTZ-induced paralysis may also involve a specific neurotoxic effect, and further studies are required to examine this possibility.

BZNT caused paralysis in cestodes with less potency than NTZ, similar to its effect in trematodes.

DNNTZ in contrast to its lack of effect in nematodes and trematodes, did cause paralysis in the cestodes. The effect was not particularly severe but does show the compound to have some anthelmintic effect.

As seen with the schistosomes, the paralytic effects of NTZ on cestodes were substantially reduced by FCS. However, for adult cestodes this is relatively unimportant since the majority of species occur in the gastro-intestinal tract and are therefore easily targeted by NTZ unbound to albumin.

5.6.2 Tegumental effect.

Within 30 minutes, NTZ caused considerable damage to the tegument of adult *H. diminuta* including blebbing, tegumental brush border abrasion and fissure formation. This suggests a tegumental targeting mechanism of action of the drug leading to impairment of structural integrity and contributing presumably to the eventual death. However, the speed in which paralysis occurred in the tapeworm was even more rapid (<5mins), than the tegumental damage suggesting that this is a secondary effect. Owing to the importance of the tegument for cestodes in nutrient absorption, the observed damage must affect nutrient uptake in a similar way to that observed with trematodes. However, death of the cestode due to impaired nutrient uptake would take significantly

longer than the time death was observed with addition of 10μg/ml (< 4 hours). Hence this is unlikely to be a primary cestodicidal mechanism of NTZ.

BZNT also caused tegument damage, characterised by the presence of deep fissures. However blebbing was not present which again distinguishes its mechanism of action from that of NTZ.

DNNTZ also caused tegumental damage, marked by slight cracking which again shows an anthelmintic effect of the compound not seen in nematodes or trematodes. As for NTZ the rapid speed in which paralysis developed with DNNTZ suggests that tegumental disruption is not the primary mechanism.

5.6.3 Protonophoric effect.

Previous work by Yorke *et al*, (1974) has demonstrated that mitochondria of *H. diminuta* are susceptible to uncoupling by protonophores. This study repeated this experiment and demonstrated significant increases in oxygen uptake by the mitochondria upon addition of 10μg/ml NTZ, TIZ, FCCP or NIC suggesting that NTZ is able to act as a protonophoric uncoupler in this system. The similarity in speed and type of paralysis amongst these compounds, further strengthens this suggestion. However, a further test should be carried out to check that the increased oxygen uptake caused by NTZ is inhibitable by the ATP synthase inhibitor, antimycin A, and could not be due to superoxide / peroxide formation. However if NTZ and TIZ are acting as protonophores against the cestodes by uncoupling oxidative phosphorylation, it remains unclear how this is directly related to a 'spastic' type paralysis, when loss of ATP would tend to prevent neurotransmission and cause a more flaccid paralysis. The possibility remains that NTZ/TIZ have direct agonist effects on cestode neuro-receptors. This could be investigated further, for instance with bungarotoxin.

5.6.4 Conclusions.

From this study cestodes are clearly very susceptible to NTZ and TIZ. Owing to the rapidity of paralysis and effect on oxygen uptake in mitochondria being similar to that of the known protonophores CCCP, FCCP and NIC, it seems likely that the primary mechanism of NTZ/TIZ is as a protonophore. NTZ also affects the cestode tegument.

Although this effect in itself would lead to death by either direct loss of worm structural integrity or by starvation owing to lack of nutrient absorption, it is unlikely that this is the primary mechanism of action owing to the rapidity that NTZ-induced mortality occurred.

BZNT also affected cestodes, but the effects were different to those observed with NTZ. This compound, like NTZ caused paralysis and tegumental disruption, though effects were not as severe as those observed with NTZ. This suggests that BZNT has either a different mechanism or has less potency against the tapeworm. DNNTZ also affected the tapeworm, however the potency was again weaker than that observed with NTZ. It is possible that this compound is acting as a protonophore (structure suggests this is a possibility), and is more active against cestodes than nematodes or trematodes perhaps due to it being more readily able to enter the tapeworm. Damage to the tapeworm tegument demonstrates a DNNTZ tegument interaction.

CHAPTER 6 – CONCLUSIONS.

The 5- nitrothiazole, nitazoxanide (NTZ) has anti-protozoal, anti-bacterial and anti-helmintic activity (Diaz *et al*, 2003; Cedillo-Rivera *et al*, 2002; McVay & Rolfe, 2000). However, the mechanisms of action of the drug are not yet fully understood. It is thought to act on bacteria and protozoa in a similar way to metronidazole, a nitroimidazole, which has an extremely broad spectrum of activity against anaerobic and microaerophilic protozoa and bacteria (Upcroft *et al*, 1999; Hardman & Limbird, 1996). Metronidazole has a low midpoint redox potential (Eo') of ≤ -400mV and is selectively toxic for organisms capable of reducing its nitro group intracellularly to a toxic nitro anion free radical, which reacts with oxygen to produce a superoxide. This can lead to the production of peroxides and the highly toxic hydroxyl radical $OH^{\bullet}$, which can cause major damage to synthetic machinery and DNA (Halliwell & Gutteridge, 1999). NTZ also has a low midpoint redox potential (Eo' ~ -350mV) and is thought to act on anaerobic and microaerophilic bacteria and protozoa in a similar way to metronidazole (Sisson *et al*, 2002). This mechanism however, does not readily explain toxicity or therapeutic ability against helminths, since they may not be able to reduce the drug. The aim of this project was to look for an alternative mechanism, which is functioning in helminths.

From this study NTZ and TIZ have shown effects on the larval and adult stages of the free-living nematode *C. elegans*, and adult *A. suum* and *H. contortus*. The effects on these nematodes were slight, characterised by varying degrees of paralysis with a wide range of potency in different species, and were not lethal. NTZ and TIZ showed no effect on the L_3 infective stage of the trichostrongylid nematodes *H. contortus* and *N. spathiger*. However, the effect of these two Romark compounds (at 10µg/ml) on trematodes (*S. mansoni* and *S. japonicum*) and cestodes (*H. diminuta* and *H. microstoma*) were more marked, characterised by rapid paralysis, tegumental damage and 100% mortality within 24 hours. Cestodes were most susceptible showing the most rapid and violent effects. Both drugs affected all life cycle stages of the trematode (except eggs) and cestodes tested, and in

comparison to known anti-trematodal and anti-cestodal drugs showed equal or greater potency *in vitro* (Table 6.1).

Table 6.1 Summary of anthelmintic activity of a) Romark compounds and b) control drugs.

a)

ORGANISM / COMPOUND	NTZ	TIZ	DNNTZ	DNTIZ	TIZg	BZNT
Ascaris suum	±	±	-	-	-	-
Caenorhabditis elegans	+	+	-	-	-	-
Haemonchus contortus	±*	±*	-	-	-	-
Nematodirus spathiger	-	-	-	-	-	-
Hymenolepis diminuta	++	++	±	±	-	±
Hymenolepis microstoma	++	++	±	±	-	±
Schistosoma japonicum	++	++	-	-	-	+
Schistosoma mansoni	++	++	-	-	-	+

b)

ORGANISM / DRUG	LEV	MEB	NIC	FCCP	CCCP	PRAZ	MET
Ascaris suum	++	N	N	N	N	N	N
Caenorhabditis elegans	++	-	-	++	++	-	-
Haemonchus contortus	++	-	-	±	±	N	N
Nematodirus spathiger	++	-	-	+	+	N	N
Hymenolepis diminuta	-	N	++	++	++	+	-
Hymenolepis microstoma	-	N	++	+	+	+	-
Schistosoma japonicum	-	N	N	N	N	++	N
Schistosoma mansoni	-	+	++	++	++	++	±

Table 6.1: Effects on worms are taken from concentrations of all drugs at 10µg/ml (*Ascaris* at 30µg/ml) after 4 hours exposure.

KEY for table 6.1

++	Highly effective	+	Moderately effective
±	Low efficacy-	-	Not effective
N	Not tested	*	Not effective against larval stage.

NTZ and TIZ are clearly most effective against trematodes and cestodes, with both showing potency equal to and sometimes greater than that seen with praziquantel. Effects with FCCP, CCCP and NIC with trematodes and cestodes are similar to those observed with NTZ/TIZ. Effect against nematodes was less severe, and in all cases levamisole

showed much higher activity. However, even a weak paralytic effect may be of great importance clinically, since this may result in worm expulsion. Of the other Romark compounds TIZg had no effect against any worms, DNNTZ and DNTIZ had slight effect against cestodes and BZNT had some effect against cestodes and trematodes but showed no anti-nematodal effect.

Several mechanisms of action are recognised for established anthelmintics. For example, levamisole acts specifically on the nematode nicotinic receptor (James & Gilles, 1985); Niclosamide is a protonophoric uncoupler (Vanden Bossche, 1985) and is also metabolised to a reductively activated nitro-compound; Mebendazole specifically inhibits the polymerisation of tubulin (Lacey *et al*, 1988). NTZ may have one or more of these mechanisms of action. The use of *C. elegans* as a convenient test system allowed the characterization of effects of NTZ upon motility. The model however, lacked the special qualities of a parasitic organism, particularly with regard to drug and nutrient uptake mechanisms. The availability of schistosomes, *A. suum* and *Hymenolepis* spp thus allowed a comparison of parasitic helminths with the *C. elegans* system, and with regards to the cestodes and the trematodes more obvious effects were seen.

6.1 NTZ as a possible neurotoxicological agent.

Initially in this study, using *C. elegans*, NTZ and TIZ, were observed to have a direct mode of action on the nematode. On addition of 10μg/ml NTZ or TIZ to *C. elegans* a primary effect characterised by spastic paralysis in 90% of the worms was observed. The paralysis took an average of 20 min to occur and was located more towards the posterior portion of the worm, characterised by increasing rigidity (characteristic of 'transient spastic paralysis' (Hardman, 1996)). This paralysis was temporary and in all cases the worms made a full recovery.

Analysis of the effects as 'transient spastic paralysis', allowed comparison with other anthelmintics which have these effects: levamisole and pyrantel. With levamisole, a known neuromuscular agent, on addition to *C. elegans*, a rapid spastic paralysis was observed. In the host, this would lead to expulsion of a parasitic nematode by gut peristalsis (James &

Gilles, 1985). Nitazoxanide clinical trials, have already demonstrated worm expulsion of cestodes (Romark, unpublished data), consistent with a paralytic mechanism. Examination of effects of NTZ on contraction of *Ascaris* muscle showed that NTZ, like levamisole, is an agonist for the parasite nicotinic receptors. However, the agonism is much weaker than that observed with levamisole presumably due to a relatively high dissociation constant for binding of NTZ to the *Ascaris* receptor. However even a weak agonist effect at receptors of a gastrointestinal worm *in vivo* could result in worm expulsion, as a high concentration of NTZ should occur in the small intestine following a 500mg or 1g dose.

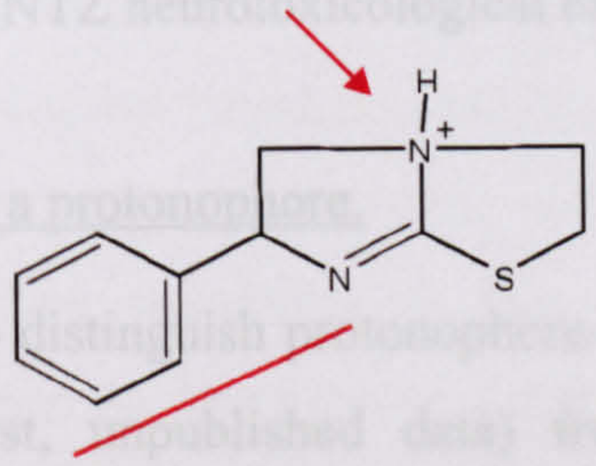
The target site of levamisole and pyrantel is the nicotinic acetylcholine receptor in nematodes (Martin, 1993), a target shared by a number of anthelmintics including morantel and bephenium. Since levamisole and pyrantel specifically affect neurotransmission at nicotinic, acetylcholine receptors, these mechanisms were investigated directly. With levamisole the nicotinic antagonists, pempidine and mecamylamine have been used to demonstrate the specificity of this drug to the acetylcholine nicotinic receptor in the nematode (Hardman & Limbird, 1996). In the presence of these antagonists, the paralytic effect of levamisole is abolished (James & Gilles, 1985). The hypothesis tested here was that if these antagonists blocked NTZ effects in *C. elegans* the mechanism of the drug might be similar to that of levamisole. Since the nicotinic antagonists pempidine and mecamylamine, indeed blocked the effect of nitazoxanide in *C. elegans*, the hypothesis is so far supported.

The detailed mechanism of action, however, still remained uncertain, as neuromuscular anthelmintics can act in different ways at the nicotinic receptor: By neuromuscular blockage, which sends waves of depolarization along the neuron to the effector organ (in this case nematode muscle) which results in a paralysis; alternatively the drugs can directly inhibit acetylcholinesterases (like one mechanism of pyrantel), which causes an accumulation of acetylcholine at the receptor, again leading to paralysis (Hardman & Limbird, 1996). Experiments performed in this study show that unlike metrifonate, NTZ does not inhibit acetylcholinesterase of *Electrophorus electricus* (although it may do so in the helminths). This suggests that NTZ is more likely to be binding to the receptor itself.

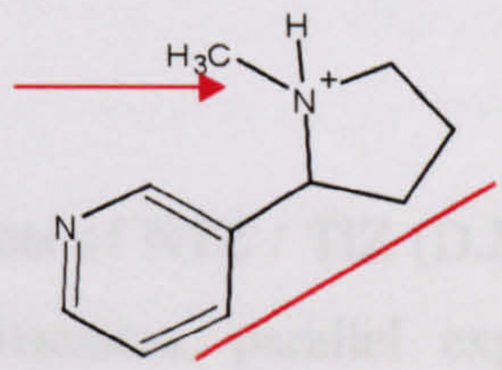
Experiments with specific mutants of *C. elegans*, insensitive to levamisole were therefore carried out to investigate nicotinic receptor activity further. Drug assays using the mutants 1072 (Unc29), 904 (Unc38) and lev 1 (nicotinic mutants and levamisole resistant) in the presence of NTZ, showed no sign of paralysis in nitazoxanide concentrations that affected 100% of the wild type, indicating the nicotinic receptor mutated in Unc38, Unc29 and lev 1 is involved in the sensitivity to NTZ. These experiments are supported using the GABA receptor mutant of *C. elegans* (407 Unc49), which, although it also had altered motility in the absence of drugs, still gave a typical response to NTZ. These results suggest that NTZ can act as an agonist at the nicotinic receptor in *C. elegans*, identical to the mechanism of action of levamisole. However, further confirmation of specific agonism should be sought using patch clamp techniques as previously demonstrated for levamisole by Martin *et al*, (1988).

Comparison of the drug structures of NTZ, nicotine and levamisole (fig 6.1), show a similarity in position of positive charges and hydrophobic area which further support the possibility that NTZ and TIZ have features similar to specific nicotinic agonists.

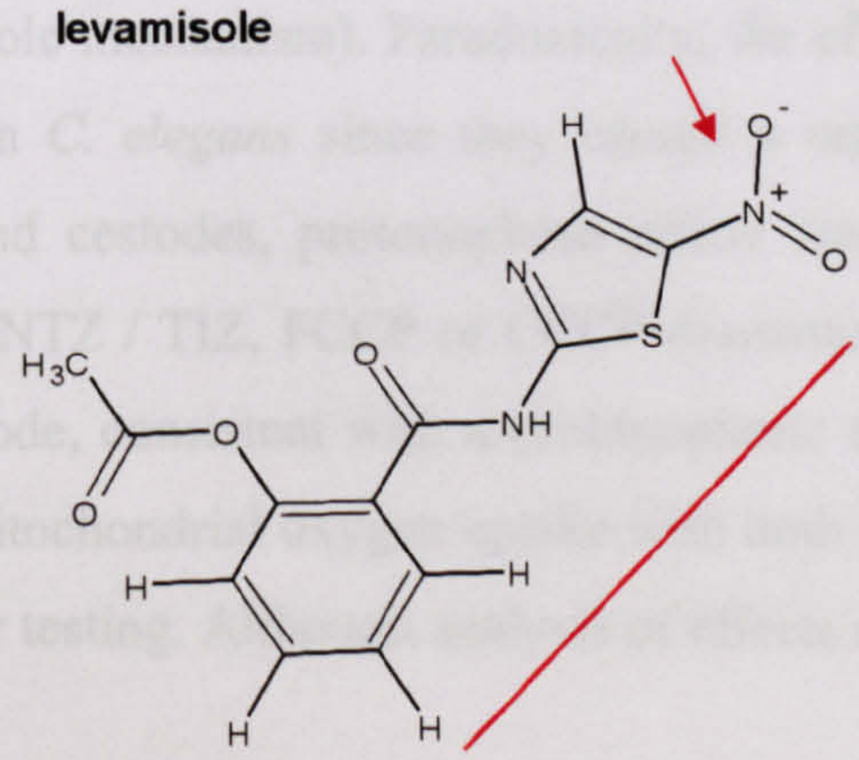
Figure 6.1 Comparison of structures of NTZ and 2 known nicotinic receptor agonists.



levamisole



nicotine



nitazoxanide

Fig 6.1: Similarity between levamisole, nicotine and NTZ:
→ **+N and hydrophobic area**

In trematodes NTZ/TIZ effects at the nicotinic receptor were examined using the nicotinic receptor agonist BTX (Tornoe *et al*, 1995) which has previously been shown by Camacho *et al*, 1995 to bind at 4° C to the nicotinic receptors on the surface of *S. haematobium*. In this study clear binding of rhBTX after incubation at 37°C was seen to the nicotinic receptors in schistosomula and adult schistosomes. Pre-treatment and post treatment with 10μg/ml NTZ/TIZ abolished or prevented this binding, suggesting direct competition at the nicotinic receptor. Alternatively, this effect could be due to alterations in receptor distribution / availability associated with changes in the tegument. Inhibition of binding however, could be due to uncoupling, where reduction of energy in the worm might prevent the uptake of rhBTX therefore preventing binding. This however seems unlikely due to the protonophore CCCP not inhibiting rhBTX binding. In such a case, energy would be reduced but rhBTX may be able to enter the schistosome passively. Clearly there is scope for more experiments involving temperature and energy related effects at the nicotinic receptors.

In cestodes direct interaction of NTZ at nicotinic receptors was not examined. However, addition of 10μg/ml NTZ to adult *H. diminuta* immediately resulted in spastic contractions of the worm suggesting a nicotinic receptor type effect. Studies need to be further extended to examine NTZ neurotoxicological effect in cestodes, for example by use of rhBTX.

6.2 NTZ as a protonophore.

In order to distinguish protonophore-dependent effects of NTZ / TIZ (D.J. Meyer and D. C. Warhurst, unpublished data) from other mechanisms, parallel experiments were generally carried out with FCCP and CCCP (and NIC – although protonophore action may not be its sole mechanism). Paradoxically, the effects of protonophores were distinct from NTZ/TIZ in *C. elegans* since they caused a rapid flaccid and tonic paralysis, but in *S. mansoni* and cestodes, protonophore action was markedly similar to that of NTZ/TIZ. Moreover, NTZ / TIZ, FCCP or CCCP dramatically reduced ATP levels in the nematode and trematode, consistent with a protonophoric effect in both cases. However attempts to examine mitochondrial oxygen uptake with both species failed probably due to insufficient material for testing. Although analysis of effects of NTZ on ATP levels was not carried out

with *H. diminuta*, an increase in oxygen uptake by isolated mitochondria was observed comparable to that seen with FCCP and NIC suggesting that uncoupling of oxidative phosphorylation occurs in this cestode. Mitochondria are abundant in the surface syncytium of cestodes (Chowdhury & Tada, 2001) especially along the basal membrane. This would allow an easy drug-induced action of NTZ and could explain the rapidity in which an effect in *Hymenolepis* spp was observed.

However the host intestinal lumen is anaerobic, suggesting that although mitochondrial electron transport and phosphorylation may still be important, one would not expect oxygen uptake to be marked. In *Ascaris*, mitochondrial electron transfer proceeds through an NADH-linked reduction of fumarate resulting in the formation of succinate (Köhler & Bachmann, 1980). This system is insensitive to cyanide and protonophores. NTZ does not inhibit fumarate reductase, but any protonophoric activity of the drug is still likely to disrupt mechanisms the organism may use to maintain cellular pH.

One discrepancy with uncoupling, is that if NTZ was acting as a protonophore it would cause a flaccid and tonic paralysis similar to that observed with FCCP and CCCP against *C. elegans*. However, NTZ caused spastic paralysis in nematodes and also caused spastic contractions in *H. diminuta* and contractions in *S. mansoni* suggesting the presence of a neurotoxicological based mechanism. However FCCP and CCCP also cause spastic and rigid paralysis in schistosomes, suggesting that they may also be targeting nicotinic receptors. One possibility is that FCCP and CCCP are being used at such high concentrations to demonstrate a weak agonistic effect for nicotinic receptors. However CCCP did not inhibit binding of rhBTX to schistosome nicotinic receptors suggesting rigidity is caused by another mechanism. One possibility is that the ATP depression due to NTZ, FCCP and CCCP, could be targeting purinergic neurotransmission which uses ATP instead of acetylcholine as a neurotransmitter (Bowman & Rand, 1990). This would explain why changes in ATP levels cause contraction and helps explain the similarity in paralysis in *S. mansoni* observed with NTZ and the protonophores (NB the ATP reduction is as predicted if there is a protonophore effect). Another factor that questions a protonophoric mechanism of NTZ, is the lack of lethal toxicity against nematodes. If a

protonophoric mechanism of action is present, death of the target organism should ensue. If NTZ is acting as a protonophore, this lack of lethal effect in nematodes could perhaps be due to antagonism by the other mechanism, for example, during paralysis there may be a much lower requirement for ATP to survive. More likely, the poor trans-cuticular entry of NTZ in nematodes (see below) presumably results in slow exposure via the digestive tract which increases the likelihood of drug metabolism / detoxication.

6.3 NTZ mode of entry.

One notable difference however, between the effect of NTZ and levamisole with *C. elegans*, is the speed in which the paralysis occurs. With levamisole, paralysis is total and occurs within a few minutes. With NTZ however, the paralysis takes longer to occur and is most evident at the posterior part of the worm. In schistosomes and cestodes the action of NTZ is very rapid. The delay in onset of paralysis in *C. elegans* may be due to the contrast between the impermeable cuticle of the nematode and the permeable absorptive tegument of the schistosome and cestode (Chowdhury & Tada, 2001) in which passive diffusion is a major mechanism of drug penetration (e.g. benzimidazoles). Thus in cestodes, lipid solubility is a major determinant factor influencing the diffusion of these anthelmintic molecules through the parasite tegument (Mottier *et al*, 2003). Presumably NTZ and TIZ are sufficiently lipophilic to be passively absorbed by the tapeworm tegument (and able to discharge directly any proton gradient which might be present). Since NTZ is less lipophilic than levamisole, it may fail to traverse the nematode cuticle and have to be ingested before any activity is observed. The rapid effects of levamisole suggest a trans-cuticular mode of entry. Further studies are required to determine the mode of entry and metabolism of NTZ in helminths. This would be facilitated by use of radioactively-labelled drug.

6.4 Damage to the tegument.

As discussed in chapter 4 and chapter 5, NTZ induces severe damage to the tegument of schistosomes and cestodes. *In vivo,* this may lead to either a lack of structural integrity, resulting in invasion by the host immune system, or more likely, a decrease in active

nutrient uptake leading to starvation of the worm. Lack of nutrient uptake was demonstrated here in *S. mansoni* with less glucose absorbed by the worm compared to the DMSO treated controls. It is also likely that this (though not tested) would affect nutrient uptake in *Hymenolepis* species as Wastling & Chappell, (1994) have already demonstrated reduced glucose uptake of *H. microstoma* after treatment with Cyclosporin A. However owing to the presence of mitochondria on the surface of cestodes (and possibly on the surface of trematodes) (Chowdhury & Tada, 2001), the tegument is undoubtedly a site of energy transduction involved in the active uptake processes. Uncoupling of these mitochondria therefore would prevent active uptake of nutrients owing to lack of energy, and it is a possibility that NTZ prevented uptake of glucose in schistosomes due to protonophoric action rather than direct tegumental disruption. However, though tegumental disruption and inhibition of nutrient uptake is a mechanism of action it might cause death to occur less rapidly than observed with the trematodes and cestodes in this study and it is therefore unlikely to be the primary mechanism.

6.5 Possible drug reduction.

In this study no evidence of NTZ reduction was observed. It was not possible to demonstrate reduction of NTZ by nitroreductases present in *C. elegans* and schistosomes. This suggests that drug-induced paralysis in these worms is not due to drug reduction, but another mechanism of action is present. However, because the organisms and the assays were not completely anaerobic and because the reduction products of NTZ have not been well-characterised, looking for a NTZ radical by loss of absorbance at a given wavelength may not have been a proper assay. Drug reduction in helminths could be looked at using electron spin resonance spectroscopy (which detects free radicals). This would require snap freezing of worms treated or untreated with NTZ and carrying out spectra in liquid nitrogen. The radical could perhaps also be characterised in an oxygen electrode using NTZ + NADPH ferredoxin reductase from spinach, + Ferredoxin + NADPH.

Drug reduction as a mechanism of activation of drug to a toxic free radical could still be a possibility in worms in *vivo*, due to the anaerobic environment in which they live being more likely to aid in NTZ reduction. This drug reduction has recently been supported by

Mansour-Ghanaei *et al*, (2003) where *Fasciola hepatica* treatment with metronidazole in Iran resulted in promising cure rates. NTZ is more easily reduced than metronidazole, and it remains a possibility that this is the mechanism of action against *Fasciola*.

6.6 Effects with other Romark compounds.

6.6.1 Denitro compounds.

It was hoped initially that use of the DNNTZ with negative results would allow proof that a nitro-group was involved in a particular toxic mechanism. However, it does not allow the conclusion of whether the nitro-group has to be reduced, or whether it is involved in neuroreceptor binding, or protonophore formation. In fact DNNTZ is likely to be a weak protonophore even though it lacks the nitro-group. Thus, only when it does have an effect on worms can useful conclusions be made: i.e. if the effect of DNNTZ is similar to that of NTZ, it cannot involve nitro-reduction. This applies to a weak paralytic effect of DNNTZ in cestodes, but its lack of lethality still leaves open the question of nitro-reduction involvement for NTZ in lethality.

6.6.2 Tizoxanide glucuronide.

No drug effect was observed with tizoxanide glucuronide in *C. elegans*, schistosomes or cestodes at concentrations used. However NTZ has a marked effect *in vivo* on *F. hepatica* infections (Romark data, unpublished), which may be due to the high accumulation of the metabolite in the bile, >114μM, which contains no NTZ and only low concentrations of TIZ (Broeckhuysen *et a*l, 2000). TIZg retains the nitro group, hence could be reductively activated if exposed to a suitable reductase, however it would be unable to traverse membranes passively because of the hydrophilic glucuronide group, and it could not have protonophore activity. There is a possibility that *F. hepatica* is ingesting TIZg and hydrolysing it back to TIZ, which would explain the presence of an effect in this parasite *in vivo*. However recent findings by Mansour-Ghanaei, *et al*, (2003) suggest that *Fasciola* can reduce metronidazole. If true, this suggests that the reductive power of the parasite *in vivo* would be capable of acting directly on TIZg.

6.6.3 BZNT.

2-benzamido-5-nitrothiazole, BZNT, showed no effect on *C. elegans*. However, in schistosomula, BZNT caused a rapid paralysis followed by death, and in adult schistosomes, it induced paralysis and caused tegumental damage, though no death of worms was observed over 120 hours. BZNT also showed activity against cestodes causing paralysis and death in the larvae, and paralysis and tegument damage in adults. In all cases, the effects, though clear, were distinct from those of NTZ. These differences must be attributable to their different chemical properties: BZNT, lacking an oxygen substituent on the aromatic ring should be less effective as a protonophore, and may be more liable to attack by amidases/peptidases which would yield the very toxic amino-nitrothiazole moiety, which is readily activated by reduction to reactive electrophilic intermediates. Furthermore, lack of the aromatic substituent would also alter any specific interaction at, for instance, the nicotinic receptor. Thus, speculatively, the likely toxicity of BZNT may be due mainly to its hydrolysis and formation of reactive electrophiles. Paralytic and protonophoric effects should be of less importance. The differences between NTZ and BZNT effects further support the conclusions of neurotoxicity and protonophore action for NTZ.

6.7 Summary.

From this study it seems likely that the mechanism of action of NTZ in nematodes is predominantly neuromuscular toxicity via specific receptor action. However, drug accessibility, metabolism and potency at different receptors yields great variability in actions on different nematodes and this would explain the differences observed in this study. In cestodes it appears likely that uncoupling of oxidative phosphorylation is occurring as a primary mechanism, though neuro-receptor drug-interaction and tegumental disruption are also occurring. Reductive activation has not been ruled out, particularly in relation to lethality. In schistosomes again a protonophoric effect is important, associated with rapid death of the worm. Studies with rhBTX clearly indicate a specific neuromuscular toxicity of NTZ, and the observed effect on the schistosome tegument and effect on nutrient absorption would serve as secondary mechanisms or even secondary

consequences of the protonophoric and neuromuscular effects. Trematodes and cestodes are most susceptible to NTZ because of the large area of absorptive tegument exposed to the external medium, in the absence of a cuticle.

The ineffectiveness of NTZ against schistosomes *in vivo* (Q. D. Bickle, Personal communication, 2000) is probably due to its high affinity for albumin (99% protein bound) and this may restrict the usefulness of NTZ to organisms which occur in relatively protein - poor compartments outside the blood, such as the gut lumen. The extent of NTZ/TIZ binding to food proteins in the gut is probably reduced on breakdown of these proteins during digestion. Investigations need to be carried out, using radioactive drug, to determine whether concentrative uptake into parasites is of importance. This may be of great significance for the *in vivo* effectiveness of any anthelmintic treatment with NTZ.

As a final statement, it can be assumed that NTZ could be demonstrating more than one mechanism of action, all aiding in its anthelmintic activity. NTZ as a protonophore would prevent energy production through electron transport and oxidative phosphorylation. During anaerobic metabolism, drug activation by nitro-reduction (not yet confirmed for this drug in helminths) might also have an impact. Finally, nicotinic receptor antagonism of NTZ, which is persistently suggested in the foregoing studies, could be an important factor in motility alteration, leading to expulsion or death of gut worms.

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APPENDIX I.

Medium 169. (Basch, 1981).

Preperation of medium 169 minus phenol red for Schistosome culture with fluorophores.

Using double strength BME 50ml (Gibco BRL) add:

0.1g lactalbumin hydrolysate
0.1g glucose
50μl hypoxanthine stock
100μl serotonin stock
100μl insulin stock
100μl hydrocortisone stock
100μl T3 stock
0.5ml MEM vitamins
5ml Schneiders insect medium
240mg HEPES
220mg $NaHCO_3$
mQ water to about 90ml
5N NaOH to pH 7.4

Filter sterilize into the original BME bottle, make up to 100ml with sterile mQ water.

E/LAC (Earle's/Lactalbumin).

Earle's Balanced Salt and Lactalbumin Enzymatic hydrolysate.

8.64g Earle's balanced salt (Sigma).
0.85g $NaHCO_3$
5g Lactalbumin enzymatic hydtolsate (Sigma).

Made up to 1 litre with sterile mQ water (pH 7.4).

Artificial Perienteric fluid. (A.P.F).

27.0g NaCl
16.0g $MgCl_2$
3.0g $CaCl_2$
3.0g KCl
5.0g Tris
5.49g Na acetate

Make up to 1 litre with de-ionised distilled water.
Warm to 37°C and make to pH 7.6 using ethanoic acid.
Add 1 teaspoon (per litre) of glucose.

LB medium.

10g Bacto-tryptone (Difco)
5g Bacto-yeast extract (Oxoid)
10g NaCl

Made up to 1 litre with deionised H_2O and autoclaved.

RPMI (Gibco)

With 25mM HEPES buffer.
Without L. Glutamine.

Hank's saline solution.

8.0g NaCl
0.4g KCl
0.14g $CaCl_2$
0.1g $MgSO_4.7H_2O$
0.1g $MgCl_2.6H_2O$
0.06g $Na_2HPO_4.2H_2O$
0.06g KH_2PO_4
1.0g Glucose
0.35g $NaHCO_3$
100mg phenol red indicator.

Make up to 1 litre with de-ionised water.
Sterilize by filtration through a bacteriological filter.

Tyrode's saline.

8.0g NaCl
0.20g KCl
0.20g $CaCl_2$
0.10g $MgCl_2$
0.05g NaH_2PO_4
1.0g Glucose
1.0g $NaHCO_3$

Make up to 1 litre with de-ionised water.

Insect Ringer Solution.

6.5g NaCl
0.14g KCl
0.12g $CaCl_2$
0.1g $NaHCO_3$
0.01g Na_2HPO_4

Make up to 1 litre with de-ionised water.

Earle's Balanced Salt solution.

NaCl - 116.4mM
KCl - 5.4mM
$CaCl_2$ – 1.8mM
$MgSO_4$ – 0.4mM
NaH_2PO_4 – 0.9mM
$NaHCO_3$ – 11.9mM
Mops – 50mM
Adjust to pH 7.2 with 5M NaOH.

Made to 1 litre with deionised water.

APPENDIX II (a) Ascariasis.

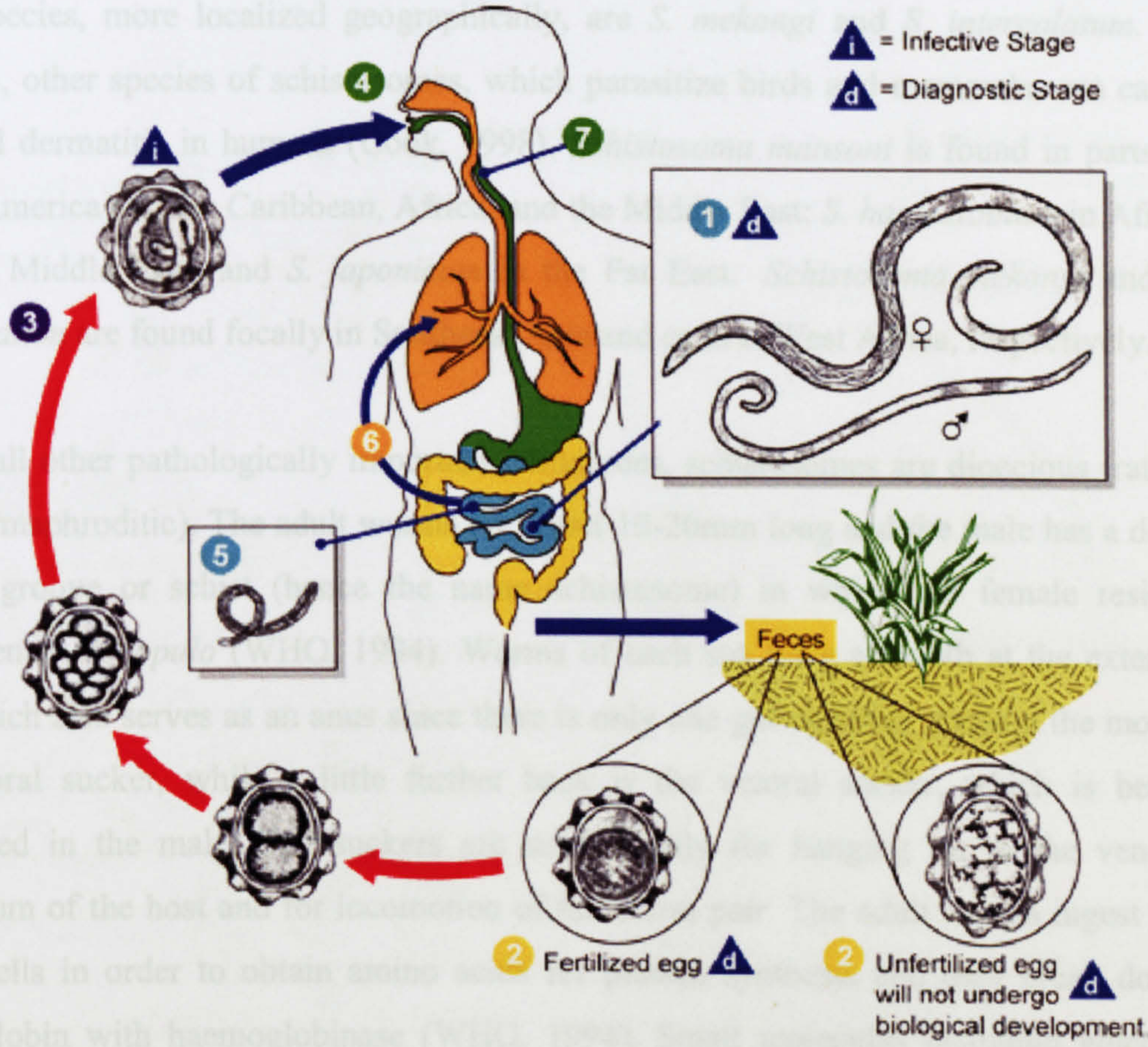
Ascaris lumbricoides is the most frequent human intestinal nematode (Georgiev, 2001). It is the causative agent of ascariasis, with an estimated worldwide prevalence of 1300-1400 million people (23% of the world population) (Muller, 2003). In humans, transmission is usually due to the hand-to-mouth route (faecal-oral) by way of contaminated agricultural products and food, or from dirty hands. It is cosmopolitan in distribution being common in both temperate and tropical countries where there are both adequate moisture and low standards of hygiene and sanitation. Although ascariasis is characterised with low morbidity and mortality rates, the global prevalence of ascariasis still results in approximately 60,000-100,000 deaths annually (Muller, 2003), primarily as a consequence of intestinal obstruction (Cook, 1998). Infection with *Ascaris* is generally asymptomatic, however, heavy infestation can sometimes cause serious pulmonary disease, or partial or complete obstruction of biliary or intestinal tracts.

Life cycle.

The adult worms (1 see digram) normally live in the lumen of the small intestine, but do not attach to the mucosa. The females (200-400mm x 3-6mm in length) are slightly larger than the males (150-300mm x 2-4mm) but may show great variation in size, depending on age and worm load (Muller, 2003). A female produces approximately 200,000 eggs (broadly ovoid and measure 45-70 μm x 35-50 μm) per day (Muller, 2003), which are passed with the faeces (2). Unfertilized eggs may be ingested but are not infective. Fertilised eggs embryonate and become infective after 18 days to several weeks (3), depending on the environmental conditions with optimal conditions being moist, warm and shaded soil. After infective eggs are swallowed (4) the larvae hatch (5) and invade the intestinal mucosa, and are carried via the portal then systemic system to the lungs (6). The larvae mature further in the lungs (10-14 days), penetrate the alveolar walls, ascend the bronchial tree to the throat and are swallowed (7). Upon reaching the small intestine, they develop into adult worms (1). Between 2-3 months are required

from ingestion of infective eggs to oviposition by the adult female. Adult worms can live 1 to 2 years.

Lifecycle of *Ascaris lumbricoides/ Ascaris suum.*



Life cycle diagram obtained from Image Library :-

http://www.dpd.cdc.gov/dpdx/HTML/ImageLibrary/Ascariasis_il.asp?body=A-F/Ascariasis/body_Ascariasis_il11.htm

The parasitic round worm *Ascaris suum* is very similar to *Ascaris lumbricoides*, causing some debate as to whether they are indeed separate species (Crompton, 2001). *A. suum* is a parasite of pigs but has been known under unusual circumstances to cause disease in humans (Georgiev, 2001). Drug treatment of Ascariasis involves using either albendazole, mebendazole, pyrantel pamoate or levamisole (Cook, 1998).

APPENDIX II (b) Schistosomiasis.

Schistosomiasis is caused by digenetic blood trematodes. The three main species infecting humans are *Schistosoma haematobium, S. japonicum,* and *S. mansoni.* Two other species, more localized geographically, are *S. mekongi* and *S. intercalatum.* In addition, other species of schistosomes, which parasitize birds and mammals, can cause cercarial dermatitis in humans (Cook, 1998). *Schistosoma mansoni* is found in parts of South America and the Caribbean, Africa, and the Middle East; *S. haematobium* in Africa and the Middle East; and *S. japonicum* in the Far East. *Schistosoma mekongi* and *S. intercalatum* are found focally in Southeast Asia and central West Africa, respectively.

Unlike all other pathologically important trematodes, schistosomes are dioecious (rather than hermaphroditic). The adult worms are about 10-20mm long and the male has a deep ventral groove or schist (hence the name schistosome) in which the female resides permanently *in copulo* (WHO, 1994). Worms of each sex have a mouth at the exterior end, which also serves as an anus since there is only one gut opening. Around the mouth is the oral sucker, while a little further back is the ventral sucker, which is better developed in the male. The suckers are used mainly for hanging on to the venous epithelium of the host and for locomotion of the worm pair. The adult worms ingest red blood cells in order to obtain amino acids for protein synthesis and they break down haemoglobin with haemoglobinase (WHO, 1994). Small molecules including glucose, amino acids, purines and pyrimidines are taken up via transtegumentary absorption. There is evidence that the female derives much of her nutrition via transtegumentary absorption from the male worm (Basch, 1991). The metabolism of adult schistosomes is largely anaerobic, by glycolysis. Adult worms live for up to 30 years with a mean life span of 3-6 years. They produce large numbers of eggs, 300 eggs per day for *S. mansoni* and *S. haematobium* and ten times this for *S. japonicum*. About one half of the eggs transit to the lumen of the urinary bladder or the intestine and leave the body in the urine or the faeces. However a substantial number of eggs are retained in the tissues where they

can survive for around 3 weeks (WHO, 1994). It is these eggs which are responsible for inducing most of the pathological manifestations of disease.

Life cycle of *Schistosoma* spp

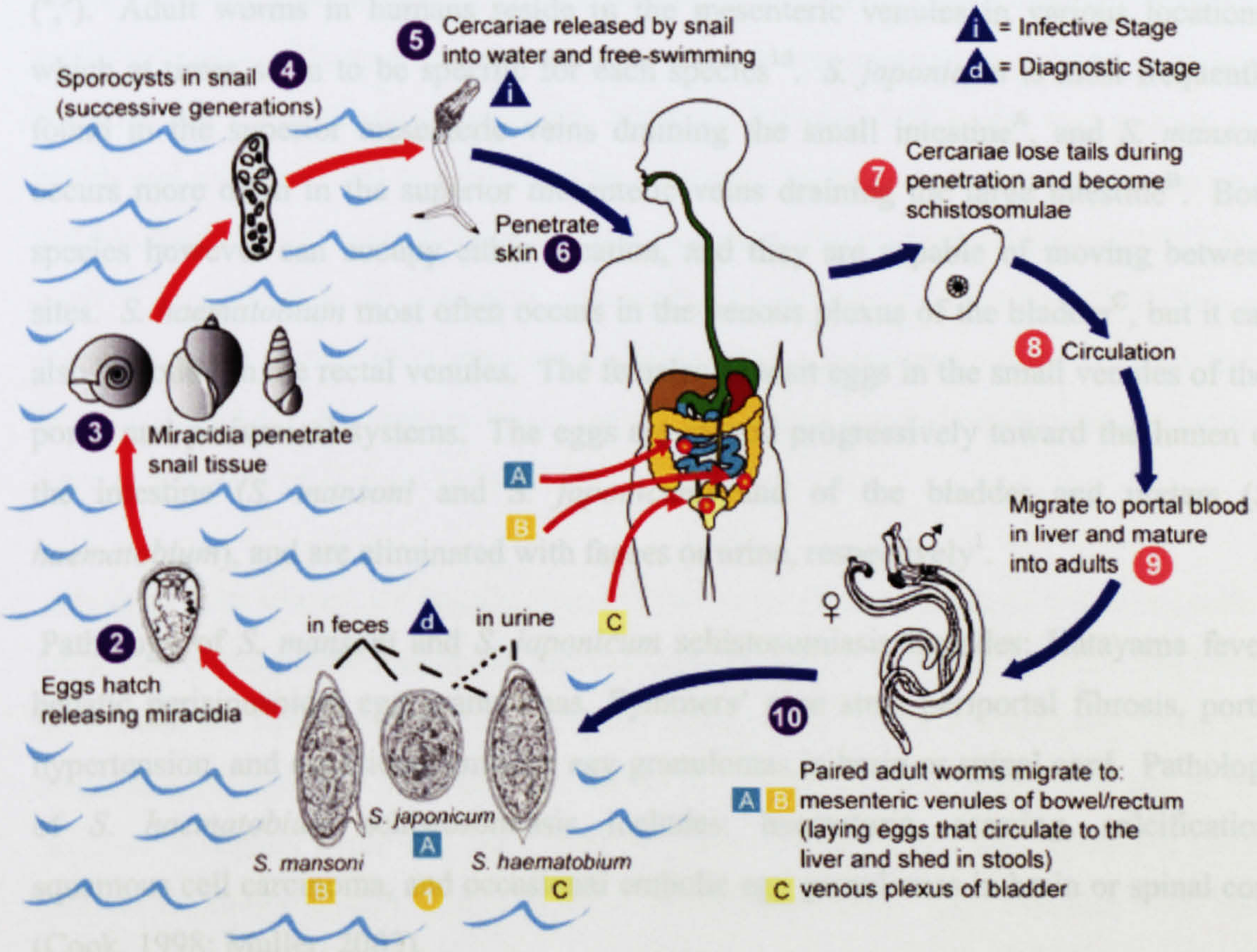


Image obtained from:
http://www.dpd.cdc.gov/dpdx/images/ParasiteImages/S-Z/Schistosomiasis/Schistomes_LifeCycle.gif

Eggs are passed out with the faeces or urine[1] and under optimal conditions the eggs hatch and release miracidia[2], which swim and penetrate specific snail intermediate hosts[3] (e.g. *Biomphalaria glabrata* for *S. mansoni* and *Oncomelania hupensis* for *S. japonicum*. The stages in the snail include 2 generations of sporocysts [4] and the production of cercariae[5]. Upon release from the snail, the infective cercariae swim, penetrate the skin of the human host[6], shed their forked tail, becoming schistosomula[7]. Once in the host, the schistosomula migrate through several tissues and stages to their residence in the veins ([8],[9]). Adult worms in humans reside in the mesenteric venules in various locations, which at times seem to be specific for each species[10]. *S. japonicum* is most frequently found in the superior mesenteric veins draining the small intestine[A], and *S. mansoni* occurs more often in the superior mesenteric veins draining the large intestine[B]. Both species however can occupy either location, and they are capable of moving between sites. *S. haematobium* most often occurs in the venous plexus of the bladder[C], but it can also be found in the rectal venules. The females deposit eggs in the small venules of the portal and perivesical systems. The eggs are moved progressively toward the lumen of the intestine (*S. mansoni* and *S. japonicum*) and of the bladder and ureters (*S. haematobium*), and are eliminated with faeces or urine, respectively[1].

Pathology of *S. mansoni* and *S. japonicum* schistosomiasis includes: Katayama fever, hepatic perisinusoidal egg granulomas, Symmers' pipe stem periportal fibrosis, portal hypertension, and occasional embolic egg granulomas in brain or spinal cord. Pathology of *S. haematobium* schistosomiasis includes: haematuria, scarring, calcification, squamous cell carcinoma, and occasional embolic egg granulomas in brain or spinal cord (Cook, 1998; Muller, 2003).

In order for infection with schistosomiasis to occur, human contact with water is necessary for infection. Some schistosome infections are zoonoses, with various animals serving as reservoirs of infection, e.g. dogs, cats, rodents, pigs, horses and goats, for *S. japonicum*, and dogs for *S. mekongi*.

APPENDIX II (c) Hymenolepiasis.

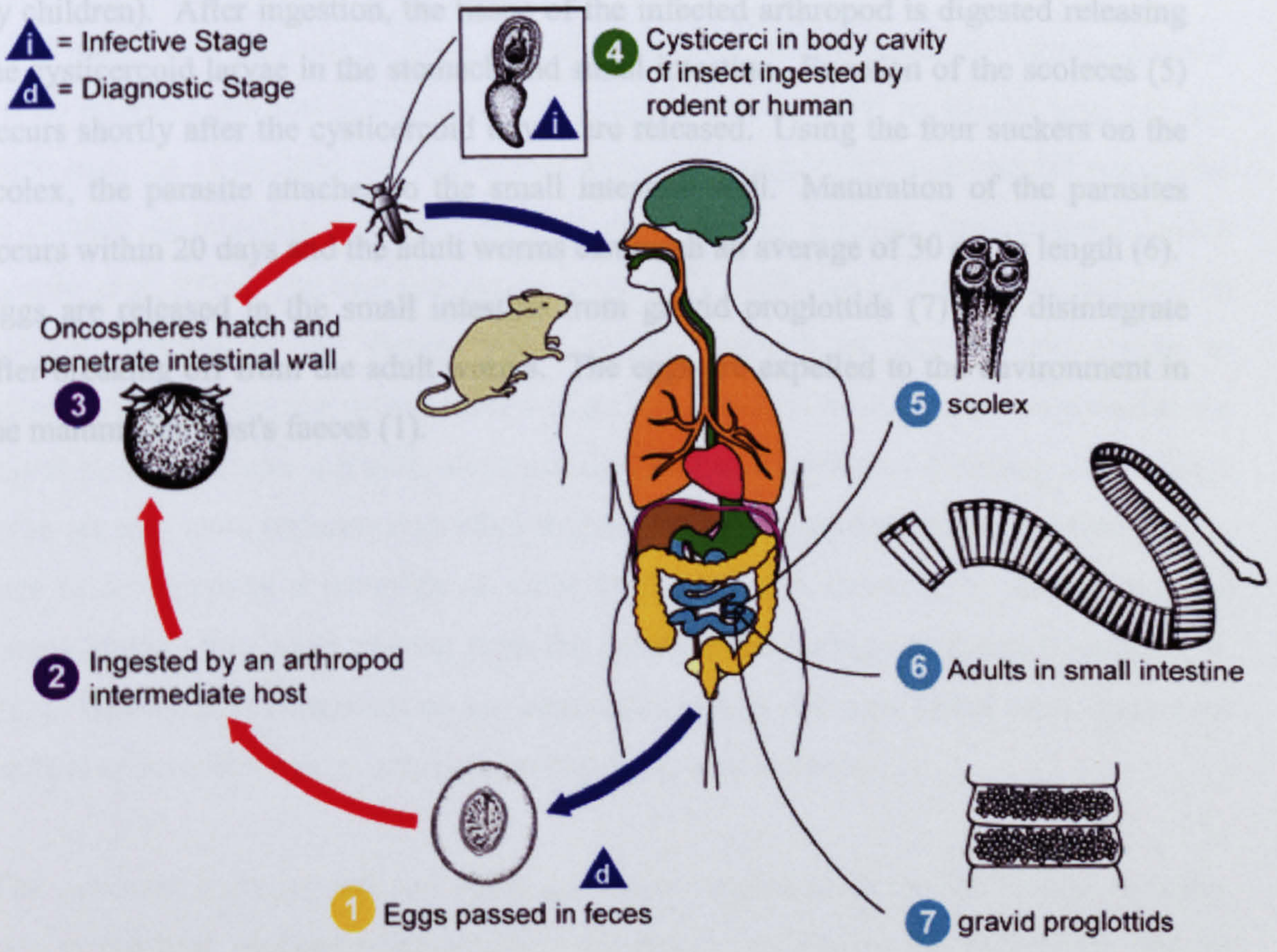
Hymenolepiasis is caused by two cestodes (tapeworm) species, *Hymenolepis nana* and *Hymenolepis diminuta*. *H. nana* (the dwarf tapeworm) is the most common cause of all cestode infections, and is of cosmopolitan distribution. In temperate areas its incidence is higher in children and institutionalized groups. *Hymenolepis diminuta* or the rat tapeworm while less frequent is a cosmopolitan parasite of rats, mice and other rodents with most cases of human infection, like *H. nana*, being reported in children (Vanden Bossche, 1985). *H. diminuta*, normally measures between 300 x 600 mm x 4mm in length and has 800-1000 proglottids. The scolex (0.2-0.4mm in diameter) is spherical and has four small suckers and a retractable rostellum; but no hooks (Muller, 2003). *H. nana* is smaller than *H. diminuta*, measuring 15-40 mm x 0.5-1.0mm and has a spherical scolex with a short retractile rostellum with a single row of 20-30 hooks and four round suckers or acetabula. There may be many thousands of tapeworms present in a heavy infection of *H. nana* (Muller, 2003).

Adults of both species are usually found in the upper three-quarters of the ileum, with the scolex usually embedded in the mucosa (Muller, 2003). However, in rats it has been shown that the tapeworm moves backwards and forwards in the intestine and this may be generally true of this and other tapeworms in humans (Muller, 2003). The egg of *H. diminuta* is slightly ovoid and has a thick yellow outer shell and a thin colourless inner membrane (embryophore), with a granular intermediate layer. Eggs measure from 60-80 μm in diameter). With *H. nana*, infection with many worms (2000+) results in enteritis, diarrhoea loss of appetite, vomiting and dizziness. With *H. diminuta*, infection in children appears to cause no clinical symptoms apart from diarrhoea. A single dose of 500mg niclosamide has been used as treatment (Muller, 2003).

Although *H. diminuta* has little direct medical importance, it is of great scientific interest as a laboratory model and is used in many research studies on the physiology, biochemistry and immunology of cestodes and for chemotherapeutic screening tests (Muller, 2003). One other tapeworm *Rodentolepis microstoma* (also known as

Hymenolepis microstoma) is also useful as a model for chemotherapeutic screening tests. *H. microstoma* is similar to *H. diminuta* but instead of a rat, matures in a mouse and has not been known to infect humans, though a recent report by Macnish *et al*, (2003) suggest the emergence of the first human case in Western Australia. Both species utilise the flour beetle, *Tribolium confusum*, as the intermediate host.

Life cycle of *Hymenolepis diminuta / Hymenolepis microstoma. .*



Life cycle diagram obtained from Image Library :-
http://www.dpd.cdc.gov/dpdx/images/ParasiteImages/G-L/Hymenolepiasis/H_diminuta_LifeCycle.gif

Eggs of *Hymenolepis diminuta* are passed out in the faeces of the infected definitive host (rodents or man) (1). The mature eggs are ingested by an intermediate host, which can be various arthropod adults or larvae (2). Species from the genus *Tribolium* are common intermediate hosts for *H. diminuta*. The cysticercoid larvae persist through the arthropod's morphogenesis to adulthood. Once ingested, oncospheres are released from the eggs which penetrate the intestinal wall of the host (3), eventually developing into cysticercoid larvae. *H. diminuta* infection is acquired by the mammalian host after ingestion of an intermediate host carrying the cysticercoid larvae (4). Humans can be accidentally infected through the ingestion of insects in precooked cereals, or other food items, and directly from the environment (e.g., oral exploration of the environment by children). After ingestion, the tissue of the infected arthropod is digested releasing the cysticercoid larvae in the stomach and small intestine. Eversion of the scoleces (5) occurs shortly after the cysticercoid larvae are released. Using the four suckers on the scolex, the parasite attaches to the small intestine wall. Maturation of the parasites occurs within 20 days and the adult worms can reach an average of 30 cm in length (6). Eggs are released in the small intestine from gravid proglottids (7) that disintegrate after breaking off from the adult worms. The eggs are expelled to the environment in the mammalian host's faeces (1).

APPENDIX II (d) *Haemonchus contortus, Nematodirus spathiger.*

Haemonchus contortus (the barber pole worm) and *Nematodirus spathiger* belong to the family Trichostrongylidae. They are of cosmopolitan distribution, though they are more prevalent in warm moist regions than in cold, dry ones. The males of both species are 10 to 20 mm and the females 18 to 30 mm long. Sheep are the usual host for *H. contortus* and hosts for *Nematodirus* spp include goats, cattle and wild ruminants.

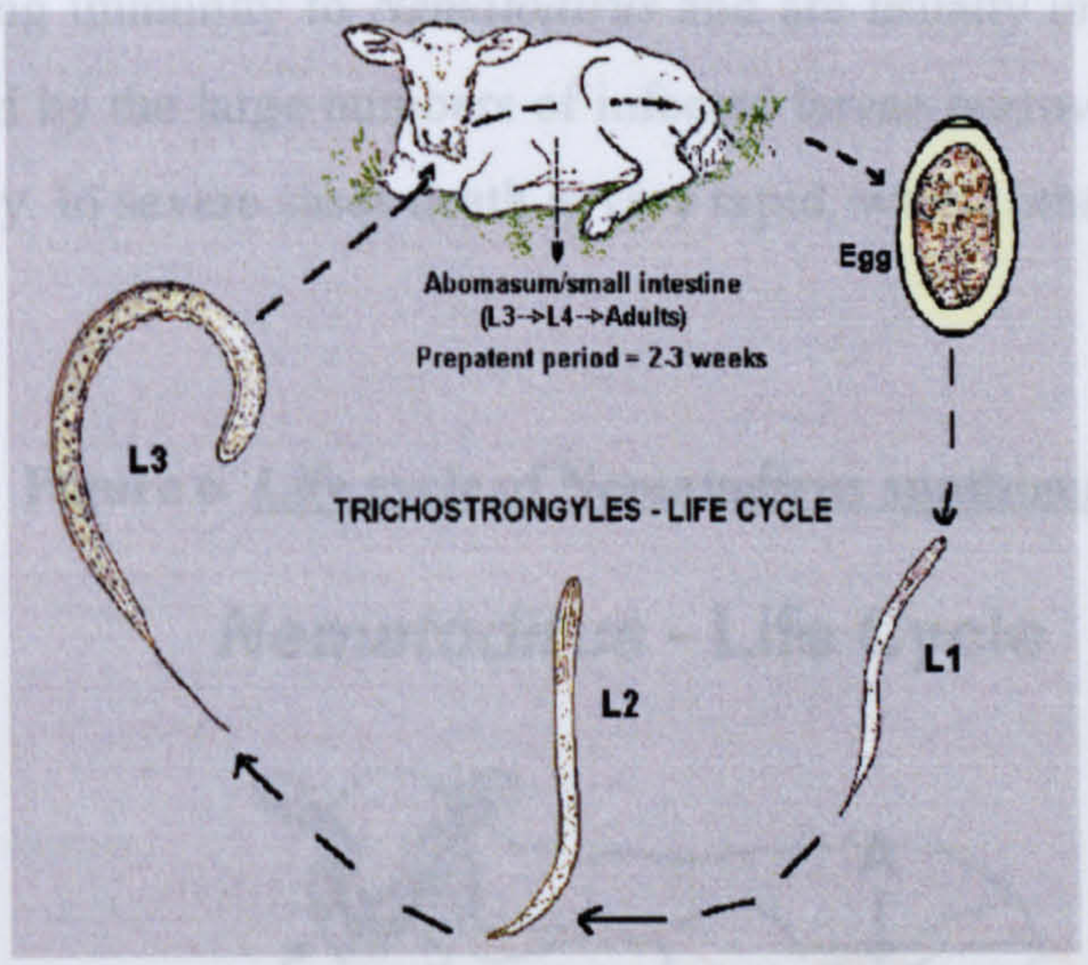
Life Cycle of *H. contortus.*

Adult male and female worms live in the abomasum (or true stomach) of ruminant animals and no intermediate host is required. The female deposits 5,000 to 10,000 eggs per day which pass out of the host with the faeces. Before the eggs of trichostrongylid nematodes are capable of infecting another host, they must develop through three stages (Vanden Bossche *et* al, 1985). The first and second stages of *H. contortus* are free-living and feed on coliform bacteria in the faeces. After varying periods of development owing to environmental factors, the infective third stage larvae (L_3) is formed. This stage retains the second stage cuticle as a sheath, does not feed and is regarded as a resting stage. These larvae are also more resistant than other stages to extremes of temperature and desiccation. Once L_3 development is complete, in order for the infective larvae to be accessible to the grazing animal, they must migrate from the faeces to the herbage (Vanden Bossche *et* al, 1985). This again is dependent on environmental factors and also faecal mass. Enormous numbers of juveniles may accumulate on heavily grazed pastures.

The ruminant becomes infected while grazing by ingestion of the third-stage juveniles. Once in the host, exsheathment occurs in the rumen, anterior to the abomasum, and the young worms pass into the abomasum where they burrow into the mucosa. Here they undergo another moult, and the fourth-stage juveniles come back into the paramucosal lumen of the abomasum. They begin to feed and undergo a final moult before reaching adulthood. Mating of adults occurs and egg production commences. Some infections may

also occur through the skin. Infection with *H. contortus* can result in emaciation, anaemia and in certain cases death.

Figure 5 Life cycle of *Haemonchus contortus.*



http://www.vetsci.psu.edu/coursedesc/vsc402/slideshows/25Trichostrongyles.pdf

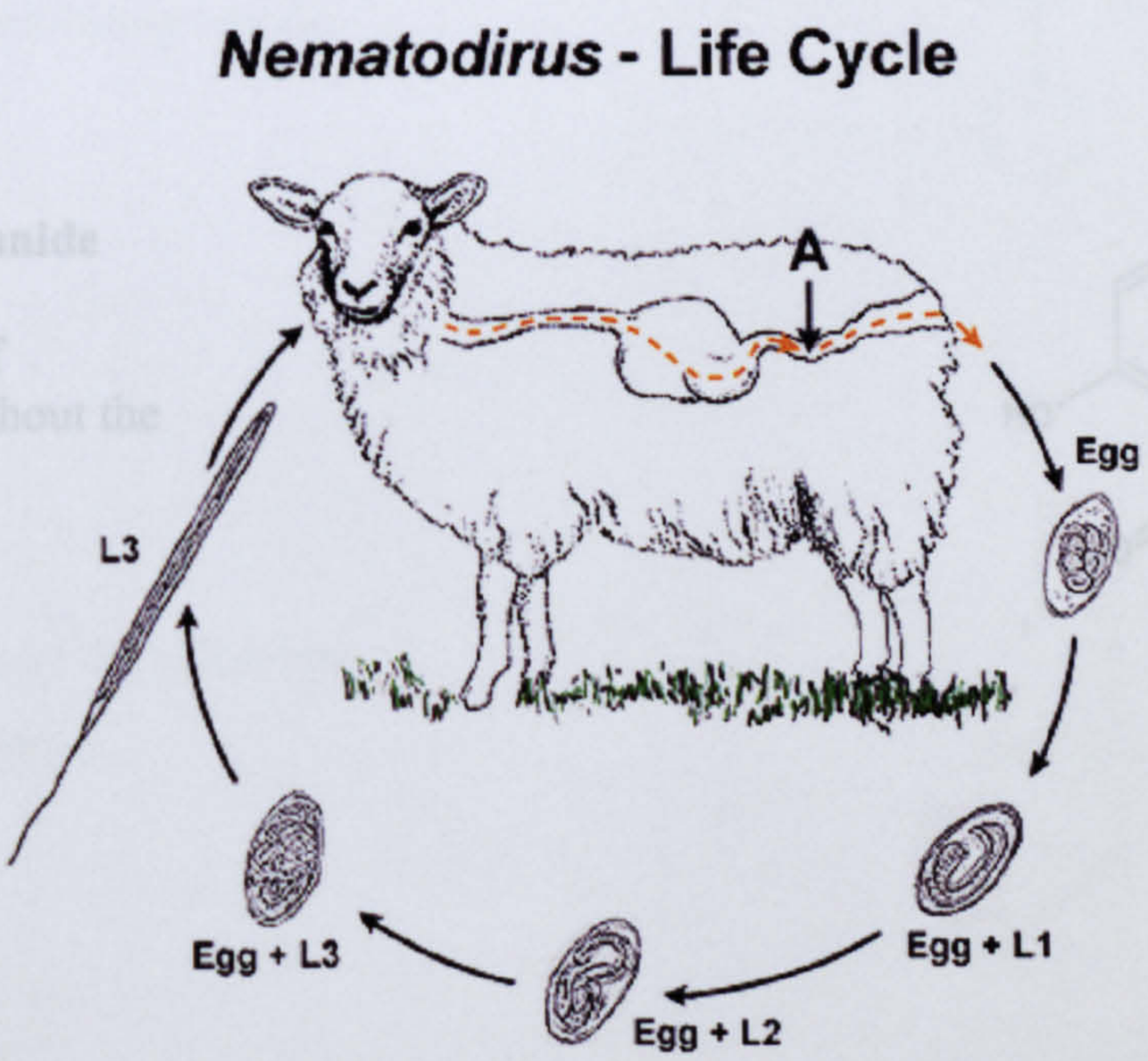
Life cycle of Nematodirus spathiger.

The life cycle of *N.spathiger* is very similar to that of *H.contortus* and other trichostrongylids, but with some important differences. The free-living development of *Nematodirus* spp differs from that of other trichostrongylids in that it is completed within the eggshell and development from egg through to the infective L_3 takes approximately one month. They are thus very resilient on the ground and able to survive winter in large numbers. Cold weather seems to prime them for spring development when the temperature exceeds 10°C and they hatch together in the spring (Vanden Bossche, 1985) . Sheep etc are infected as with *H. contortus* by ingestion of the infective larvae whilst grazing. Following ingestion of L_3's, exsheathment occurs in the abomasum and subsequent developing stages are found on the mucosal surface of the small intestine. The parasitic phase is non-migratory and the pre-patent period is 15 days.

Nematodirus is not usually a primary pathogen in ruminants in North America and its importance derives from an additive effect in mixed infections of nematodes causing

parasitic gastroenteritis. However, *Nematodirus* does cause significant disease in lambs in Britain. This is because its unusual hatching requirements may result in heavy contamination of pastures with newly hatched L_3's just as lambs are close to weaning and are beginning to graze pastures more in late spring (May and early June in Britain). Diarrhoea followed rapidly by dehydration are the primary clinical signs. Adult ewes have developed a strong immunity to *Nematodirus* and are usually unaffected. The damage to the lamb is caused by the large numbers of infected larvae burrowing into the lining of the gut simultaneously. In severe cases death is very rapid, whilst other lambs may take several days to die.

Figure 6 Life cycle of Nematodirus spathiger.

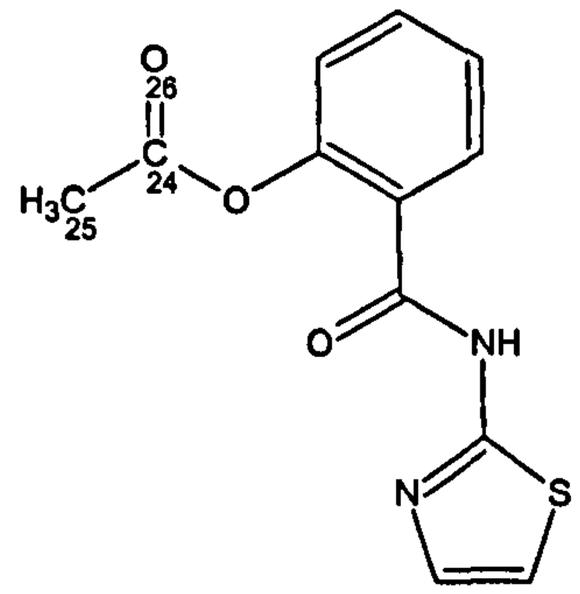


http://cal.nbc.upenn.edu/merial/Trichos/images/nemdir_c.gif

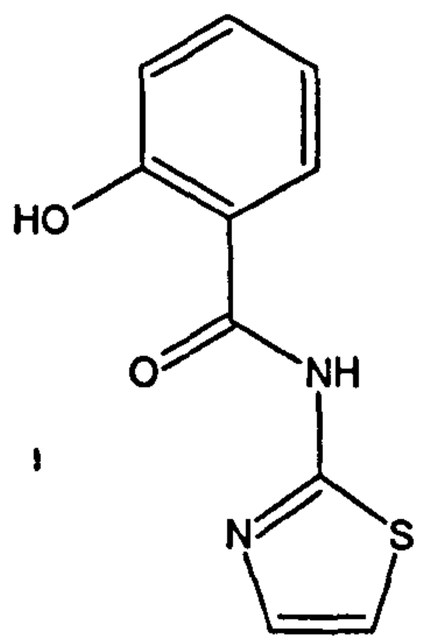
Presently trichostrongylid infection represents a major economic scourge. Control is by drug therapy typically included in the animals feed. Resistance however, is a problem with documented cases of anthelmintic resistance reported in benzimidazole compounds and ivermectin (Prichard, 1994).

APPENDIX III - Drug Structures.

Denitro-nitazoxanide
(DNNTZ)
A derivative of nitazoxanide, without the nitro-group.

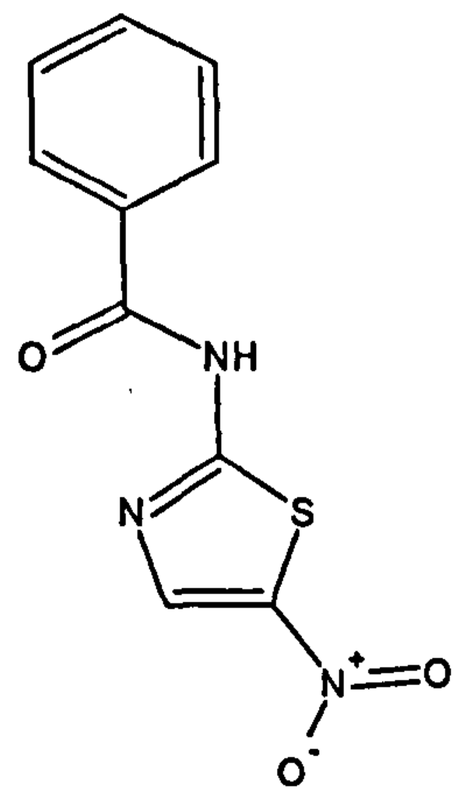


Denitro-tizoxanide
(DNTIZ)
A derivative of tizoxanide, without the nitro-group.



2-benzamido-5-nitrothiazole
(BZNT)
A derivative of tizoxanide.

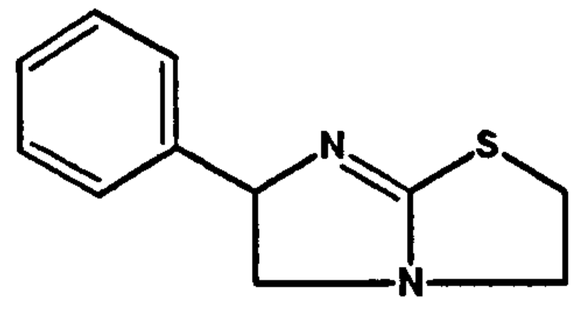
benzamido nitro thiazole



Levamisole.

(LEV)

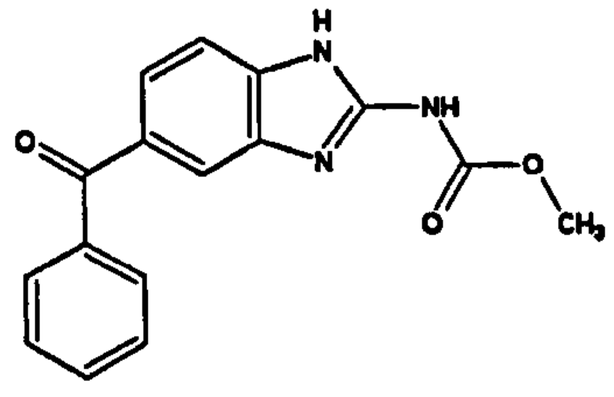
Anti-nematodal drug. Acts on helminth nicotinic receptors.



Mebendazole.

(MEB)

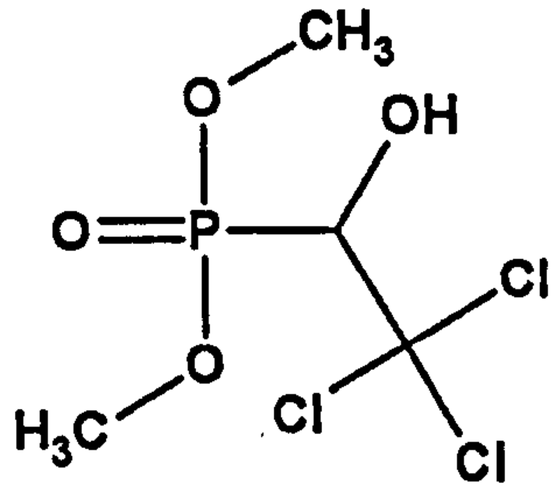
Anti-nematodal benzimidazole drug. Acts by inhibiting tubulin polymerisation.



Metrifonate.

(MET)

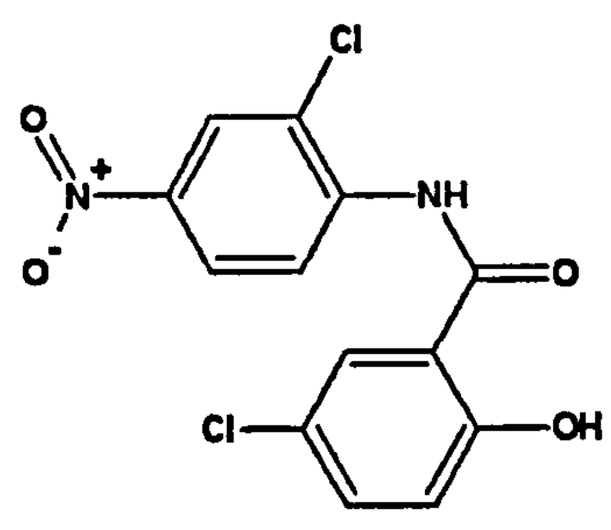
Anti-schistosomal drug. Inhibits acetylcholinesterase



Niclosamide.

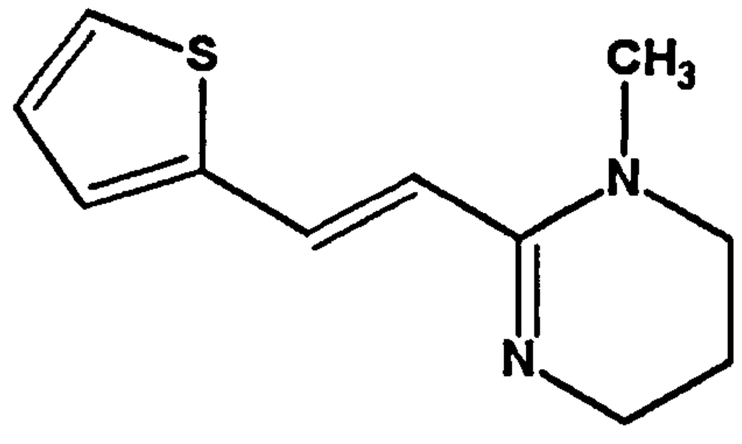
(NIC)

Anti-cestodal drug. Protonophore. Uncouples mitochondrial oxidative phosphorylation.



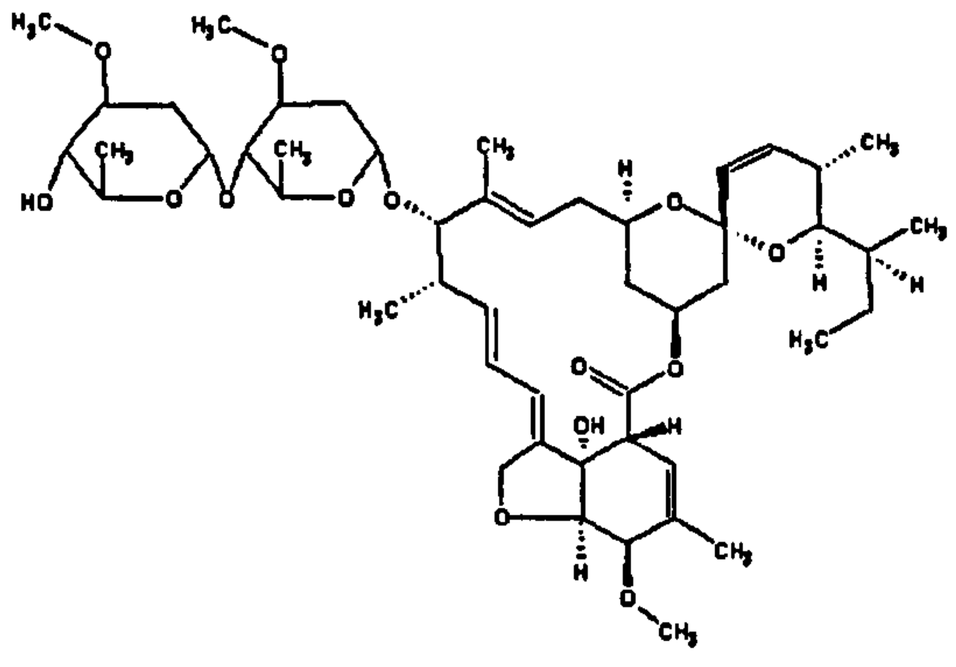
Pyrantel.

Anti-nematodal drug. Acts on helminth nicotinic receptors. Also inhibits acetylcholinesterase.

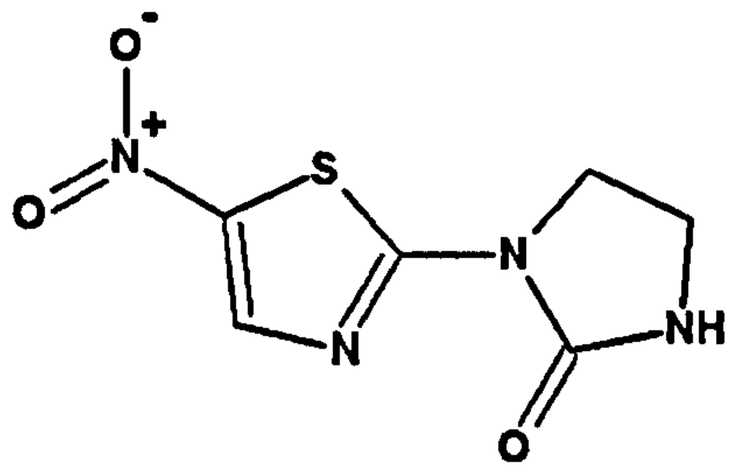


Avermectin a

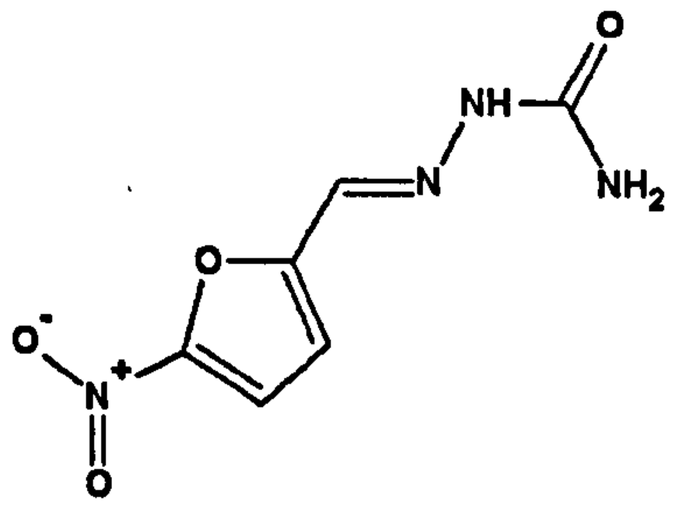
Anti-nematodal drug. Acts on helminth GABA receptors.



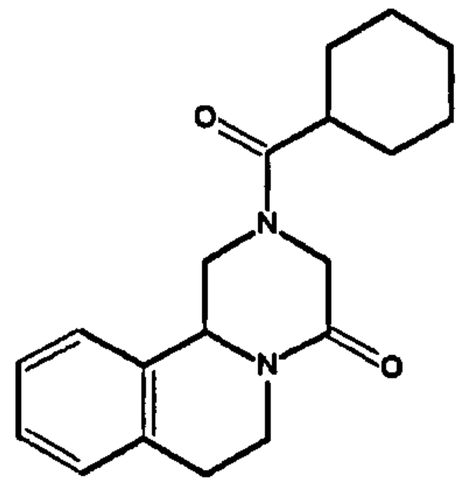
Niridazole.
(NIRI)
Anti-schistosomal drug. No longer in use due to mutagenic properties.



Nitrofurazone
(NF)
Antibiotic. Anti-trypanosomal drug.

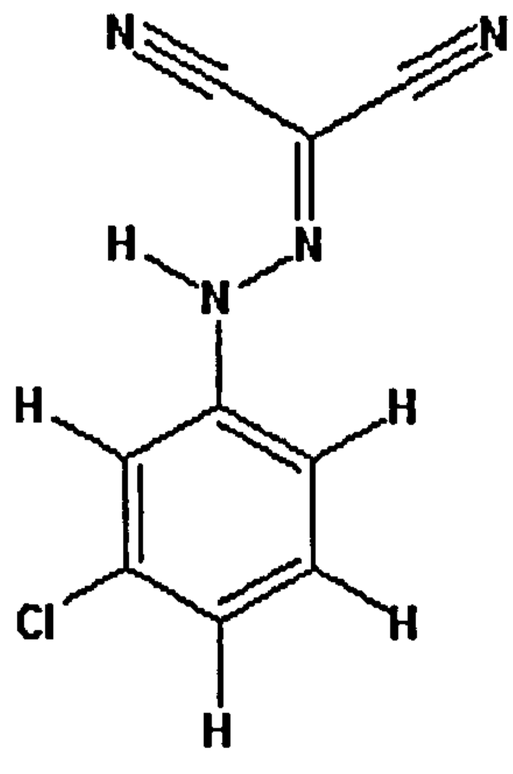


Praziquantel.
(PRAZ)
Anti-trematodal drug. Drug of choice for treatment of schistosomiasis. Also has anti-cestodal activity.



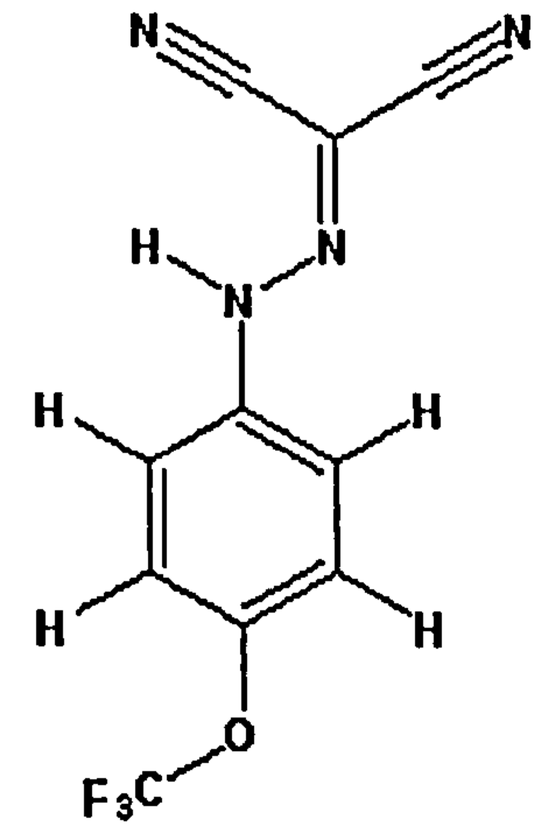
CCCP.

Protonophore. Uncouples oxidative phosphorylation.

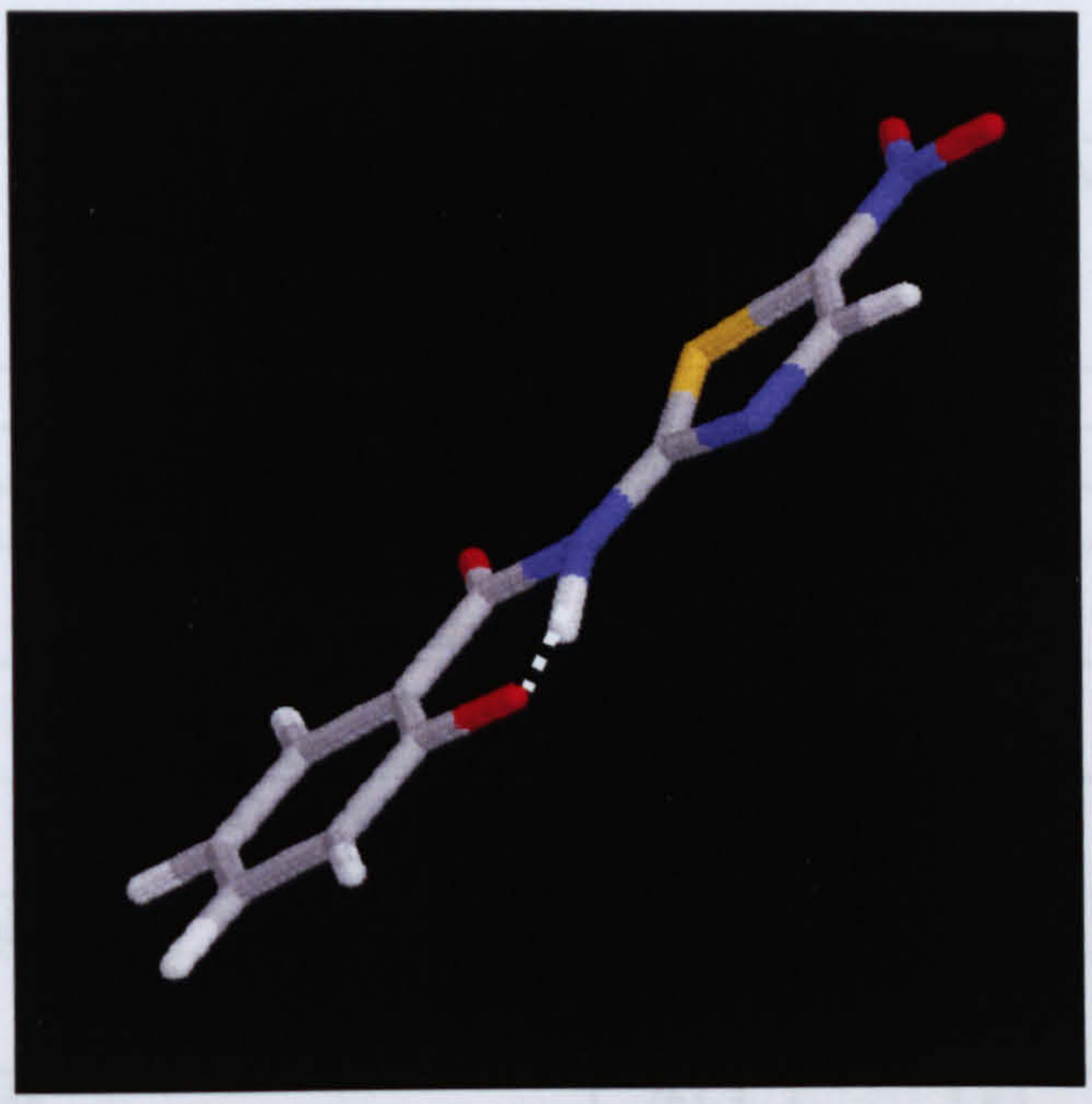


FCCP.

Protonophore. Uncouples oxidative phosphorylation.



A crystallographic study of tizoxanide carried out by Dr. John Lisgarten and Prof Rex Palmer of Birkbeck College, University of London.



The above crystallographic study shows the two rings of tizoxanide are completely coplanar, and linked by an H-bond between >N-H and the phenolic -OH effectively giving a 3-ring structure.

APPENDIX IV

Molecular weight / Molarity Data on Romark Test Compounds & Related Drugs

Compound	Other name	Mol Wt	Molarity (μM) of 1μg/ml soln	Molarity (mM) of 10mg/ml stock
Nitazoxanide		307.3	3.25	32.5
p-Nitazoxanide		307.3	3.25	32.5
m-Nitazoxanide		307.3	3.25	32.5
Tizoxanide	Deacetyl-Nitazoxanide	265.2	3.77	37.7
p-Tizoxanide	Deacetyl-p-Nitazoxanide	265.2	3.77	37.7
m-Tizoxanide	Deacetyl-m-Nitazoxanide	265.2	3.77	37.7
Tizoxanide-glucuronide		441.4	2.27	22.7
2-Benzamido-5-Nitrothiazole	Deoxy-Tizoxanide	249.2	4.01	40.1
Denitro-Nitazoxanide		261.3	3.83	38.3
Denitro-Tizoxanide		219.2	4.57	45.7
Metronidazole		171	5.84	58.4
Niclosamide		327	3.06	30.6
Mebendazole		295	3.86	38.6
Praziquantel		312.4	3.2	32.0
FCCP	Carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone.	254.2	3.93	39.34
CCCP	Carbonylcyanide m-rophenylhydrazone	204.6	4.89	48.88
Ivermectin		269.8	3.71	37.06
Oxamniquine		279.3	3.58	35.8
Metrifonate		257.4	3.89	38.85
Lucanthone		340.5	2.94	29.37
Hycanthone		356.5	2.81	28.05

APPENDIX V.

From the *Caenorhabditis elegans* genome, a member of the oxygen-insensitive nitroreductases which strongly resembles the FMN linked nitroreductase from *Enterobacter cloacae* was found by (Bryant & DeLuca *J Biol Chem* 1991 5;266:4119-25). It is possible that this enzyme may be able to reduce NTZ causing a toxic effect in *C. elegans*.

A consensus sequence was obtained from sequences of nitroreductase from various species using ExPASy (http://ca.expasy.org/) (D. C. Warhurst, unpublished data)

```
                    10        20        30        40        50        60
                ....*....|....*....|....*....|....*....|....*....|....*....|
consensus     1 DVLELITTRRSIRKFD-DKPVSEEELEEILEAARLAPSSGNLQPWHFIVVEDEELKEKLA 59
C.elegans   146 iFYEEMKMRRSCRQFS-SRDVPLKVIQNLLKTAGTSPSVGNLQPWTFCVVSSDSIKTMIR 204

                    70        80        90        100       110       120
                ....*....|....*....|....*....|....*....|....*....|....*....|
consensus    60 ELAGG-----QRQVESASAVIVVCADTNRAQSALKLYG----------GRVEEILDAARD 104
C.elegans   205 KILEAd---eRDNYVSRKKGASWVVDVSQLQDTWRRPY--------------ITDAPYL 246

                   130       140       150       160       170       180
                ....*....|....*....|....*....|....*....|....*....|....*....|
consensus   105 LSIAAVNAAVAAESLGRGFDWESKQSY-IAVGNLLLAARALGLDSCPISGFDLGKPAERL 163
C.elegans   247 LIVCHEIFRDVHSKTERVFHYNQISTS-IAVGILLAAIQNVGLSTVVTSPLNAGPDISRi 305
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